# Les effets des facteurs de stress environnementaux sur les poissons côtiers : approche expérimentale et in situ

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# THE EFFECTS OF ENVIRONMENTAL STRESSORS ON COASTAL FISH:

# *IN SITU* AND EXPERIMENTAL APPROACH

Présentée et soutenue publiquement

par

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# Abstract

Estuaries and coastal areas are essential fish habitat as nursery and spawning but characterized by the presence of multiple interacting stressors, both natural and anthropogenic, which can represent potential threat toward aquatic organisms, especially for commercial fish species. In this context, the impacts of environmental stressors such as chemical contamination and Harmful algal blooms (HABs) have been studied by *in situ* and experimentally (microcosm and mesocosm) approaches on two fish species: European flounder (*Platichthys flesus*) and European sea bass (*Dicentrarchus labrax*). During this thesis, we used various indicators to determine fish responses to environmental stressors. Among these tools, we used the growth and condition indices, molecular biomarkers and immunological parameters.

*In situ* approach has been realized in two different systems anthropogenically influenced: one is heavily impacted system (Seine estuary) and the others are less impacted and/or considered as ''clean" systems (Canche, Authie and Somme estuaries). As juvenile flounders concentrate in estuaries, we have chosen this species as a biological indicator to evaluate the quality of these estuarine habitats. This *in situ* study emphasized the negative impact of contaminants on the nursery function of estuaries. The Seine estuary exhibited the highest metals and PAHs contents in sediment compared to other estuaries and metal concentrations in juvenile flounder of this estuary were also significantly higher than ones collected in the less polluted estuaries. In the same way, fish growth and condition indices were significantly lower in individuals from this estuary in spite of the sufficient food availability. To control environmental parameters such as hydrological parameters and food availability, a microcosm experiment was carried out on sea bass juveniles exposed to fresh sediment from five sites with different chemical concentrations using multi-biomarker approaches. After 21 days exposure, no metal accumulation in fish gills and any significant differences on the physiological performances and immune system responses of fish juveniles could be observed. On the other hand, responses of molecular biomarkers, particularly, EROD, GST and CAT activities increase with the chemical contamination gradient after 7 days of exposure in sediment. This microcosm study confirmed the sensibility and relativity of short term molecular biomarkers responses to the chemical contamination. These two studies highlighted the complexity of the fish responses to environmental stressor due to the many variable environmental factors *in situ* and due to the selection of fish species (pelagic or benthic) and the exposure duration in controlled laboratory assays.

Beside the impact of pollution on fish, Harmful algal blooms (HABs) are widespread along the Eastern English Channel and may alter ecological functions of coastal zones and thus affecting nursery grounds and fish populations. Nevertheless, the effects of two recurrent harmful algal blooms: a) *Phaeocystis globosa* and its degraded form transparent exopolymeric particles (TEP) with foam accumulation and b) *Pseudo-nitzschia pseudodelicatissima* (exponential versus senescent phase) was investigated on the growth and condition of sea bass juveniles. Both mesocosm experiments exhibited any negative impact on juvenile sea bass physiological performance, hence, their survival and recruitment success. In conclusion, the results of this thesis contributed to improve the fish responses with multi-biomarker approaches to monitor and assess the health of fish communities and fish habitat quality, as well as the general ecological status of coastal zones and estuaries against the various environmental stressors.



Keywords: Pollution, harmful algal blooms, fish, multi-biomarkers, fish growth, condition, estuaries

# Résumé

Les estuaires et les zones côtières constituent des territoires à forts enjeux stratégiques économiquement et pour l'environnement. Ils assurent de nombreuses fonctions biologiques et écologiques dont celle de nourricerie et de frayère pour les poissons. Ces écosystèmes sont pourtant soumis à de multiples facteurs de stress, à la fois naturelles et anthropogéniques, qui peuvent représenter une menace potentielle envers les organismes aquatiques, en particulier pour les espèces commerciales de poissons. Dans ce contexte, les effets de facteurs de stress environnementaux tels que la contamination chimique et les efflorescences algales nuisibles (HABs) ont été étudiés par des approches *in situ* et expérimentales (microcosme et mésocosme) sur deux espèces de poissons: le flet (*Platichthys flesus*) et le bar (*Dicentrarchus labrax*). Durant cette thèse, nous avons utilisé différents indicateurs pour déterminer les réponses des poissons aux stress environnementaux. Parmi ces outils, nous avons utilisé des indices de croissance et condition, des biomarqueurs moléculaires et des paramètres immunologiques.

Une approche *in situ* a été réalisée sur deux systèmes différents en termes d'influence anthropogénique: l'un est un système fortement impacté (estuaire de la Seine) et les autres sont des systèmes moins impactés et/ou considérés comme "propres" (les estuaires de la Canche, Authie et Somme). Comme les juvéniles de flet se concentrent dans les estuaires, nous avons choisi cette espèce comme indicateur biologique pour évaluer la qualité de ces habitats estuariens. Cette étude *in situ* a souligné l'impact négatif des contaminants sur la fonction de nourricerie des estuaires. L'estuaire de la Seine a montré les concentrations les plus élevées en métaux et HAP dans les sédiments par rapport aux autres estuaires et les concentrations en métaux mesurées dans les juvéniles de flet de cet estuaire ont également été significativement plus élevées que ceux échantillonnés dans les estuaires moins pollués. De même, la croissance des poissons et les indices de condition ont été significativement plus faibles chez les individus de cet estuaire en dépit d'une disponibilité de nourriture suffisante. Pour contrôler les facteurs environnementaux tels que les paramètres hydrologiques et la nourriture, une expérience en microcosme a été réalisée sur les juvéniles du bar exposés à des sédiments frais prélevés sur cinq sites avec différentes concentrations en contaminants en utilisant des approches multi-biomarqueurs. Après 21 jours d'exposition, aucune accumulation de métaux dans les branchies des poissons et différence significative sur les performances physiologiques et les réponses du système immunitaire des juvéniles de poissons n'ont été observées. Par contre, les réponses des biomarqueurs moléculaires, principalement, les activités d'EROD, GST et CAT augmentent avec le gradient de contamination chimique des sédiments après 7 jours d'exposition. Cette étude en microcosme confirme la sensibilité et la relative réponse précoce des biomarqueurs moléculaires par rapport à la contamination chimique. Ces deux études ont souligné la complexité des réponses des poissons aux stress environnementaux du fait des nombreuses variables environnementales *in situ* et de la sélection d'espèces de poissons (pélagique ou benthiques) et de la durée d'exposition dans les essais en laboratoire.

Outre l'impact de la pollution sur les poissons, les efflorescences algales nuisibles (HABs) sont fréquentes le long de la Manche orientale et risquent d'altérer les fonctions écologiques des zones côtières et affecter ainsi les zones de nourricerie et par conséquent des populations de poissons. Néanmoins, les effets de deux proliférations d'algues nocives récurrentes: a) *Phaeocystis globosa* et ses dégradés telles que les particules transparentes exoplymeriques (TEP) ainsi que la mousse résultant du bloom d'algue et b) *Pseudo-nitzschia pseudodelicatissima* (en phase exponentielle par rapport à la phase sénescentes) ont été étudiées sur la croissance et la condition des juvéniles du bar. Les deux expériences en mésocosme n'ont pas montré d'impact négatif sur les performances physiologiques des

juvéniles de bar et par conséquent sur leur survie et le succès du recrutement. En conclusion, les résultats de cette thèse ont contribué par l'utilisation combinée de plusieurs approches et de différents biomarqueurs à améliorer la connaissance sur les réponses des poissons face aux divers facteurs de stress environnementaux, ainsi que l'évaluation du statut écologique des zones côtières et estuariennes.

Mots-clés: Pollution, efflorescences algales nuisibles, poisson, multi-biomarqueurs, croissance et indice de conditions des poissons, estuaires

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# List of scientific publications

**Filipuci, I**., Henry, F., Courcot, L., Billon, G., Amara, R., 2010. Performances physiologiques des juvéniles de flet *(Platichtyhs flesus*) en réponse à la contamination métallique dans différents estuaires de la Manche Orientale. *Journal des jeunes océanographes (accepted).*

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# Posters

**Filipuci, I.** and M. Toğulga, 2007. Preliminary investigation on the biological characteristics of red mullet (*Mullus barbatus* L., 1758) from Çandarlı Bay (Northern Aegean Sea) ; XII European Congress of Ichthyology, Cavtat-Crotia; 9-13 Septembre.

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# Oral communications

**Filipuci, I.,** Henry, F., Curcot, L., Billon, G., Minier, C. and Amara, R., 2010. Physiological performances of juvenile European flounder to contamination in different estuaries in the Eastern English Channel. ECSA (Estuarine & Coastal Sciences Association) 47 Symposium, Figueira da Foz –Portugal, 14-19 Septembre.

# CHAPTER I

# GENERAL INTRODUCTION

## I.1. Coastal zones and its importance

Aquatic ecosystems are essential components of water resources and in the well being of all living organisms. They perform many important environmental functions such as recycling of nutrients, purifying water, attenuating floods, recharging ground water and provide habitats for wildlife. Aquatic ecosystems are also used for human recreation, and are very important to the tourism industry, especially in coastal regions. Coastal ecosystems are complex systems that play a major ecological role and their importance and preservation have been highlighted in recent decades, particularly following the adoption of several conventions or international guidelines (European projects BioMar[1], BEEP[2], European organizations CIEM[3], OSPAR[4], WFD[5] etc.). Coastal ecosystems provide a wide array of goods and services: they host the world's primary ports of commerce; they are the primary producers of fish, shellfish, and seaweed for both human and animal consumption; and they are also a considerable source of fertilizer, pharmaceuticals, cosmetics, household products, and construction materials. Transition systems such as estuaries filter pollutants from inland freshwater systems, and help to protect shorelines from erosion and storms (Burke et al., 2001). On the other side, the continental shelves play an important role in the oceanic uptake of atmospheric $CO_2$, even though it only represents 7% of the total oceanic area (Padin et al., 2007).

The coastal zone is a transitional area directly influenced by the characteristics and usage of the adjacent land (Gazeau et al., 2004). Those are the regions of remarkable biological productivity and high accessibility and provide the major source of protein for 1-2 billion people of the poorest parts of the Earth (Kullenberg, 2010).

BioMar[1], Biological Markers of environmental contamination in marine ecosystem.
BEEP[2], Biological Effects of Environmental Pollution in marine coastal ecosystems.
CIEM[3], International Council for the exploration of the Sea.
OSPAR[4], Oslo-Paris Convention, which aims to conserve marine ecosystems and to protect human health in North-East Atlantic, by preventing and eliminating pollution, protecting the marine environment against the adverse effects of human activities, and contributing to the sustainable use of resources.
WFD[5], The European Water Framework Directive which aims to achieve by 2015 a good ecological status for all aquatic environments and preserve those in good condition.

They provide also considerable benefits to society while at the same time human activities exert pressure on coastal ecosystems, therefore threatening those same benefits (Nobre, 2009). Within these zones, the coastal waters are of ecological importance for several reasons, perhaps the most important being that they support 25% of global primary production and 80% of global carbon production. In addition, they sustain major socioeconomic activities, such as tourism, agriculture and fisheries (Flo et al., 2011). Globally, more than 3 billion people live in proximity to the marine coast and the population intensity along the coastal region increases pressure on the utilization of resources leading to habitat destruction, degradation and fragmentation, fisheries, mariculture operations, shipping, dredging, land reclamation, the discharge of sewage and industrial chemicals as well as more subtle, unintentional effects associated with diffuse sources of marine pollution. Nonetheless, the exponential growth of human population and progressive industrialization are posing serious threats to aquatic environment and its resource potential (Bowen and Depledge, 2006).

Coastal environments are characterized also by the presence of multiple interacting abiotic stressors, including changes in temperature, oxygen concentration and environmental salinity (Schulte, 2007). Over the last 100 years, these habitats have also undergone a remarkable degree of anthropogenic environmental change, subjecting the organisms that live in these already challenging habitats to an increasingly complex pattern of environmental stressors. Both global and local anthropogenic factors are having and will continue to have substantial impacts on these habitats. For example, oceans were previously considered to be a vast reservoir for the safe disposal of pollutants. Many chemical contaminants, including organochlorine compounds, herbicides, domestic and municipal wastes, petroleum products and heavy metals are now recognized to have adverse affects on ocean environments, even when released at low levels (Haynes and Johnson, 2000; Pinto et al., 2003). Little attention has been given to this problem until shortly before the 19th century. The adverse effects of environmental pollution have been well documented in recent years (Hiss et al., 1999; Swaminathan, 2003). Nonetheless, marshes and estuaries are expected to be particularly strongly affected by global warming and associated sea level increases (Scavia et al., 2002), and many estuaries have also been highly modified as a result of factors acting at local scales (Nichols et al., 1986) because of the high human population in coastal areas around the world (Vitousek et al., 1997). Direct human modification of coastal lands and waters also continues to increase. Lotze et al. (2006) showed patterns of species and habitat loss, species invasions, and water quality degradation in estuaries and coastal seas around the world. Another

example, the increasing prevalence of fin- and shellfish aquaculture in coastal waters has significantly modified their nutrient regimes in some cases, either through the direct introduction of nutrients in food or the removal of nutrients with harvest (e.g., Yamamuro et al., 2006). Increased port development and shipping activity associated with the growth of trade has affected coastal water quality in and near ports, including the dredging of channels for increasingly large cargo vessels (Wolanski, 2006). Wastes from both industrial and domestic sources have also a substantial impact on the coastal environments (Moore et al., 2004). Internationally accepted procedures for environmental/ecological impact and risk assessment have been established to manage human impact on coastal environments (Rice, 2003).

The major components of these coastal waters are shallow coastal habitats and estuaries which present a very high level of productivity (Whittaker, 1975; Costanza et al., 1997). Because of some of their features, such as high salinity variations, low depths, muddy grounds, warm water, higher turbidity, the presence of various and rich habitats, high food availability and refuge from predators, these zones provide for the growth and survival of young fish and serve also as spawning nursery grounds for many marine fish species (Gibson, 1994; Meng et al., 2002; Le Pape et al., 2003; McLusky and Elliott, 2004; Elliott et al., 2007; Franco et al., 2008). In contrast with their ecological importance, estuaries area amongst the most modified and threatened aquatic environments (Blaber et al., 2000). However, these coastal areas are densely populated with about 45-50% of the global population living in an about 100 km broad band from the sea; in some countries going up to 100% of the population (Coleman et al., 2008; Diaz and Rosenberg, 2008). Furthermore, estuaries are the discharge point for all particles stemming from anthropogenic activities carried out within the drainage basin, including urban and industrial development as well as intensive agriculture. Therefore, in addition to increasing quantities of nutrients and organic materials, estuarine waters and sediments accumulate xenobiotics such as heavy metals and organic contaminants, which tends to degrade the quality of the remaining estuarine habitats for juvenile fishes. In addition, habitat degradation is one of the most serious threats for the recovery of marine organisms such as fish stocks (Hall, 1998). As a consequence, the essential nursery function of coastal habitats may be reduced by these quantitative and qualitative factors related to anthropogenic disturbances (Gibson, 1994; Able et al., 1999; Costa and Cabral, 1999; Phelan et al., 2000; Meng et al., 2001; Jones et al., 2002; Whitfield and Elliott, 2002; Gilliers et al., 2006; Coates

et al., 2007; Le Pape et al., 2007). Recruitment level and population size of the concerned marine species may then be dramatically affected (Peterson et al., 2000).

The increase of human activities along rivers, estuaries and in coastal areas affects ecosystems, in particular by pollution and habitat destruction (Coleman et al., 2008; Diaz and Rosenberg, 2008; Halpern et al., 2008). Hence, due to the development of more industries, increased urbanization and reclamation of areas, the environment is under severe stress which results in the degradation of the quality of the environment and create an unfavorable condition for aquatic organisms. In these ecosystems, organisms are exposed to a combination of environmental stressors such as physical or chemical pollutants as well as other stressors such as parasites and environmental impact (e.g. climate change or habitat loss). The combination of stressors can result in increased risk to organism (either additive or synergistic effects) or decreased (protective or antagonistic effects). During the past two decades, the public has become increasingly concerned with hazardous and toxic materials discharged into the aquatic environment. The toxic materials may accelerate detrimental effects either directly or indirectly on the marine organisms in question. It is well established that pollutants of various kinds reach the aquatic environments either accidentally or deliberately and may be found in tissues of aquatic vertebrates and invertebrates (Han et al., 1997) and then cause acute and/or chronic effects such as death, malformations, increased susceptibility to disease, and eventually the disappearance of the fish species. The pollutants or other stressful situations can weaken the organisms making them susceptible to disease or they cause disease directly. Another example, land-use practices and the resulting introduction of nutrients into estuaries and coastal environments have caused increased frequency of harmful algal blooms, and episodes of prolonged aquatic hypoxia (Diaz and Rosenberg, 1995; Conley et al., 2002; Gray et al., 2002; Rabalais et al., 2002). Another source of pollution, marine oil pollution has been receiving increasing attention since the middle of the 19th century with the intensification of tanker operations and oil use (Islam and Tanaka, 2004), marine tanker collisions (Owen, 1999), pollutant release from coastal refineries (Wake, 2005; Tolosa et al., 2005) and continuous operative discharges from ships (ESA, 1998; Carpenter and MacGill, 2001). Annually, 48% of the oil pollution in the oceans is due to fuels and 29% to crude oil. Tanker accidents contribute only 5% of all pollution entering into the sea (Brekke and Solberg, 2005). Despite this, an estimated 1.6 million tons of oil have spilled from tankers since 1965 (over 650,000 ton in Europe and Pacific Asia) (Wang and Fingas, 2003). The worst oil spill disasters in the history are those of *Amoco Cadiz* in 1978 from the coast of

Brittany, France with about 277 000 t oil spilled (1.6 million barrels) (NOAA, 1978; Bellier and Massart, 1979); *Exxon Valdez* in 1989 in Prince William Sound, Alaska with 35 500 t (Galt et al., 1991); *Erika* in 1999 in the bay of Biscay, France with 19 800 t (Le Guerroue et al., 2003); *Prestige* in 2002, 46 kilometers away from the Finisterra Cape, in the Northwest of Galicia, Spain with 63 000 t (Albaiges et al., 2006); *Deepwater Horizon* in 20$^{th}$ April- 15$^{th}$ July 2010 in the gulf of Mexico, USA with 678 000 t (Houck, 2010); *ExxonMobil* oil spill in May 2010 in Niger Delta, Nigeria with 95 500 t (http://www.adn.com, 2010) and *Xingang* Port oil spill in 16-21 July Yellow sea, China with 90 000 t (http://www.voanews.com, 2010). After the drama of *Amoco Cadiz* oil spill in 1978, the French government set up a monitoring plan, "POLMAR Plan" (Polution Maritime), in case of accidental pollution of the marine environment, allowing mobilization and coordination of control methods appropriate to the type of pollution. Moreover, the Centre of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE) is a national expert who assists the administrative authorities in charge of the fight against accidental pollution and coordinates with the expertise provided by other agencies likely: IFREMER (French Research Institute for Exploration of the Sea), Meteo France, SHOM (The Hydrographic and Oceanographic Service of the Navy).

The coastal zone is increasingly being used for a diverse array of often conflicting uses. Recent approaches to coastal management and protection have highlighted the need to base management practices on a detailed understanding of the processes at work within the coastal zone (Baily and Nowell, 1996). Scientists can aid in the environmental management of these conflicts by providing high-quality technical information to decision-makers, yet in scientifically valid forms. In recent years this challenge has been met through the use of multi-metric index approaches, that have been developed for simplifying the use of extensive ecological data (Cooper et al., 1994; Boesch, 2000; Ferreira, 2000; Paul, 2003), and with the development of indicators as management tools to address environmental issues (Belfiore, 2003; Aubry and Elliott, 2006).

The European Union has adopted several environmental directives, strategies, recommendations, and agreements that require a shift from local- or regional-based regulations to more ecosystem-based, holistic environmental management. Over the next decade, environmental management in Europe is likely to focus more on biological and ecological conditions rather than physical and chemical conditions, with ecosystem health at the center of regulation and management decision making. Successful implementation of this

new ecosystem management and strategic assessment process in Europe will require the integration of regulatory and technical information and extensive collaboration from among European Union member countries, between agencies, and across disciplines to an unprecedented degree. It will also require extensive efforts to adapt current systems of environmental assessment and management to the basin and ecosystem level, across media and habitats, and considering a much broader set of impacts on ecosystem status than is currently addressed in most risk assessments. This will require the understanding, integration, and communication of economic, ecological, hydrological, and other processes across many spatial and temporal scales (Apitz et al., 2005).

In this context, they have been signed many international, national and European agreements for the protection and management of coastal zones. For example, the implementation of the European Water Framework Directive (WFD; 2000/60/EC) establishes the guidelines for water resources management with well defined objectives for the protection of groundwater, inland, estuarine and coastal waters. This framework requires Member States to assess the Ecological Quality Status of transitional and coastal waters by 2006 and achieve at least good ecological status in all water bodies by 2015. The WFD outlines that Member States must collect information on the type and magnitude of significant anthropogenic pressures, and identify in specific cases Heavily Modified Water Bodies. Moreover, Integrated Coastal Zone Management (ICZM) concerned with the planning and management of resources within the coastal area, across the range of habitats and land use types, including land and water management. It relates the management of resources to particular pressures upon the coastal zone and the human activities which take place there (including fishing, tourism, urban and industrial development) as well as the importance of such areas as sites for the conservation of natural habitats and species. On 24 October 2005 the European Commission (EC) proposed a 'Thematic strategy on the protection and conservation of the marine environment' (COM (2005)504). The overall aim of this strategy is to achieve a good environmental status of European marine waters by 2021 and to protect the resource base upon which marine-related economic and social activities depend. This strategy is a key component of the Green Paper on Maritime Policy of the EC, announced in the Strategic Objectives of the EC for 2005-2009. The Urban Waste Water Treatment Directive (UWWD) (91/271/EEC, adopted in 1991, amended in 1998) has the aim to ensure that all significant discharges of sewage (public services and industrial sectors) undergo treatment before discharged into surface waters, estuaries or coastal waters. This includes waste water

collection and treatment for all settlement above 2,000 population equivalents, biological (secondary) treatment, and nutrient removal (tertiary treatment) where the receiving waters show an elevated nutrient level and/or eutrophication. The North Sea Ministerial Conferences (NSMCs) aim to provide a political framework for the intensification of work within relevant international bodies, and also to ensure more efficient implementation of the existing European and international rules related to the marine environment in all North Sea States.

OSPAR and Paris conventions for the protection of the marine environment of the North-East Atlantic aim to regulate and control marine pollution. Furthermore, The Ramsar Convention's mission is "the conservation and wise use of all wetlands through local, regional and national actions and international co-operation, as a contribution towards achieving sustainable development throughout the world. Nevertheless, the MARPOL convention is the main international convention covering prevention of pollution of the marine environment by ships from operational or accidental causes. MARPOL was adopted on 2 November 1973 at the International Maritime Organization (IMO) (and modified by the Protocol of 1978) and covered pollution by oil, chemicals, harmful substances in packaged form, sewage and garbage.

## I.2. Context of the Eastern English Channel

In the European seas, the English Channel, a coastal sea in the northeastern Atlantic, represents an interesting transition zone between the temperate and boreal regions. The English Channel is an epi-continental sea with a megatidal regime and subjected to heavy anthropogenic pressures (Figure 1). It constitutes an important northwestern European crossroads, both in economic and bio-geographical terms. Its shallow eastern basin (< 50 m) is home to many new maritime activities, including energy production (e.g., thermo-nuclear power plant, offshore wind mill), marine resource exploitation (e.g., aggregate extraction), and increased portuary activity (e.g., greater capacity for container unloading). Furthermore, the English Channel is regarded as commercially vital area both high level of shipping traffic and other economic activities. Biologically the area has also a high biodiversity value (Birchenough et al., 2010).

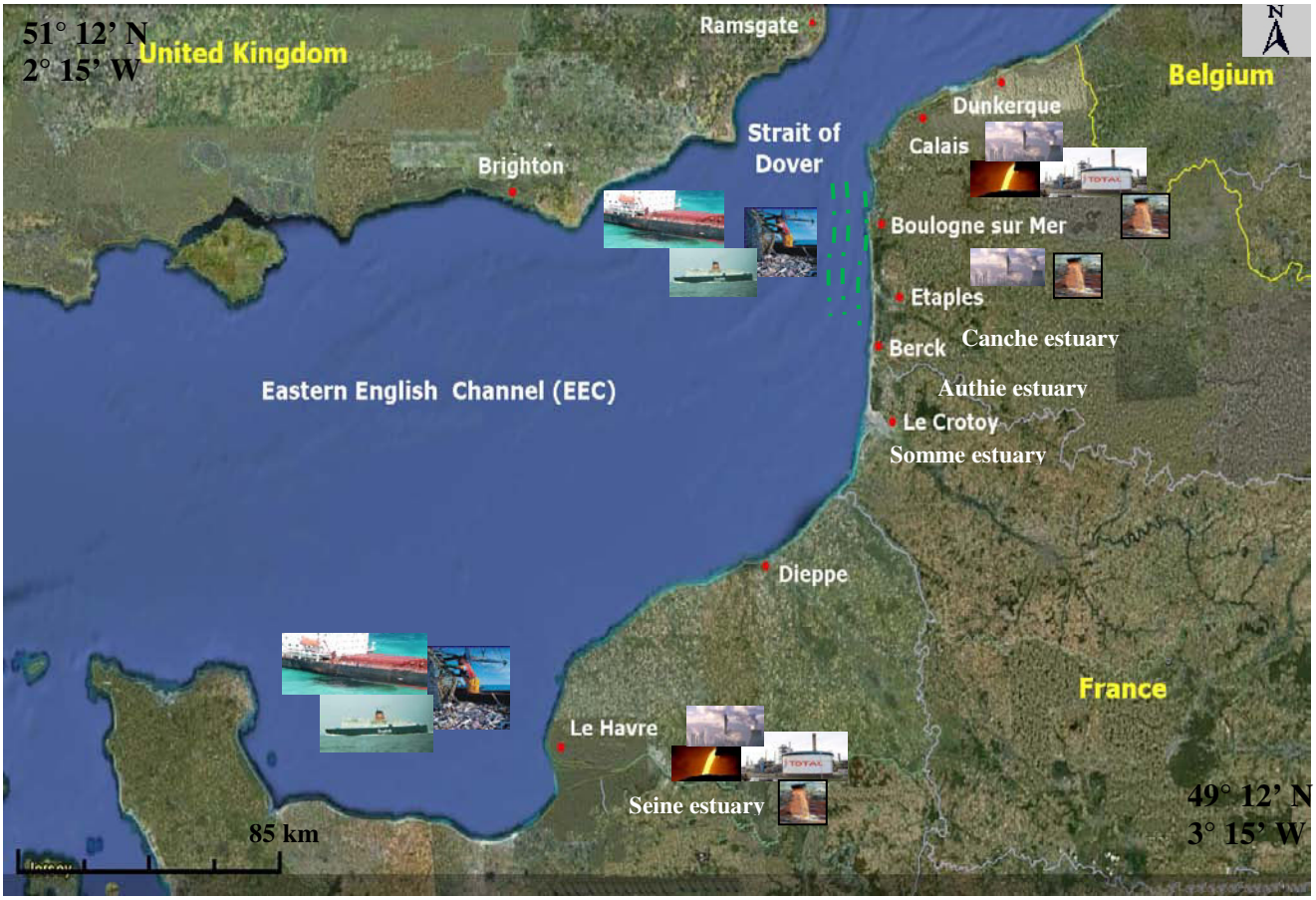



Figure 1. Principals' location of anthropogenic pressures along the Eastern English Channel

The English Channel is one of the world's busiest straits for maritime shipping, allowing vessels to travel between the Atlantic Ocean and North Sea (20% of global maritime traffic). The English Channel is an economic key area for other numerous activities, such as leisure and tourism, international ports and the exploitation of living resources (e.g. fisheries, shellfish farming) or mineral aggregates (e.g. sand and gravel). It is one of the areas containing the majority of aggregate resources for the construction industry. This area is also significant for fisheries because of the abundance of many commercial fish species and migratory routes linked to specific environmental characteristics (Martin et al., 2009; Birchenough et al., 2010). The inshore waters of the Eastern Channel support nursery areas

for several commercially important species, especially the common sole (*Solea solea*, L.), plaice (*Pleuronectes platessa*, L.), sea bass (*Dicentrarchus labrax*, L.) or the sprat (*Sprattus sprattus*, L.) (Cefas, 2008; Amara et al., 2007, 2009; Rochette et al., 2010). The vulnerable living resources and their habitats are subjected to strong anthropogenic pressure (e.g. from fisheries, mineral extraction, offshore wind farms, pollution threats from maritime accidents, etc.) (Martin et al. 2009). This anthropogenic pressure has led to the progressive deterioration of the environmental quality of the milieu, particularly at the mouth of the Seine estuary (Dauvin and Lozachmeur, 2006). The Seine estuary is the largest megatidal estuary in the English Channel and, as such, is economically important for France, with 25% of France's population as well as 40% of its industry and agriculture concentrated in and around it (Dauvin, 2007). In the southern North Sea, water quality is also influenced by large industrial complexes surrounding Calais, Dunkerque, Boulogne and Le Havre harbours. A large variety of manufacturing industries, primarily metallurgical, chemical and petrochemical, are responsible for the input of a range of contaminants of varying significance (Dewarumez and Davoult, 1997). In contrast, the area from Dieppe to the Canche estuary is free from major industrialization, although water quality may be impacted by contaminants discharged from the Seine River (Desroy et al., 2003). Another natural disturbance in the eastern English Channel is the *Phaeocystis spp.* bloom which is one of the most recurrent phytoplankton blooms recorded in the northwest European shelf seas and can represent 80 % of total phytoplankton abundance in spring (e.g. Breton et al., 2006; Schapira et al., 2008; Grattepanche et al., 2011). Although high biomass blooms of *Phaeocystis* may cause serious ecological and economical problems with their harmful effects on the environment and biota (Lancelot et al., 1987; Masó and Garcés, 2006) there is no information on their effects on ichthyofauna.

### I.3. The quality of ecosystems and effects on organisms; the use of biological indicators

In many coastal and nearshore marine areas, human activities introduce distinctive pollutants whose introduction into the natural environment can produce severe alterations in the different trophic levels of the ecosystems. Therefore, it is essential to protect these habitats and develop necessary monitoring methods using biological indicators to estimate their quality. Nevertheless, the link between the quality and health levels of ecosystems, environmental/ecotoxicological risk, ecological stress, and public health concerns are

established recently, but not particularly well understood from a mechanistic point of view. This unsatisfactory situation is driven less by the lack of interest in, or general insights about these relationships, than by a concern that acquisition of sufficient information is beyond the available technical and financial resources of those who need to know (Bowen and Depledge, 2006).

Selection and development of biological indicators that can distinguish anthropogenic effects from natural variability is at the heart of ecosystem habitat quality assessment. Nevertheless, this habitat quality cannot be measured directly and can only be described on a comparative basis. Bioassesment studies used for this kind of studies shows that a combination of both rapidly responding and sensitive biomarkers and the more ecological relevant bioindicators should be incorporated in filed bioassessment designs (Figure 2).

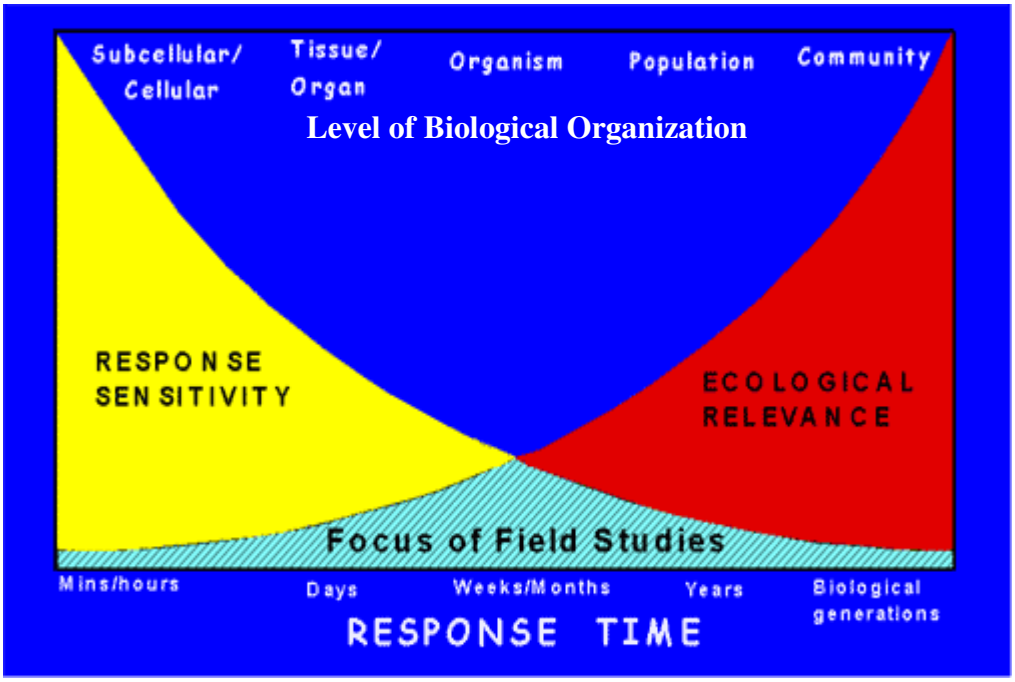



Figure 2. Scheme about the organism-level responses in the field studies related on the biomarkers and ecological relevant bioindicators (Adams, 2002)

In this context, a variety of approaches has been developed to evaluate the health of ecosystems and organisms, ranging from the community level to cellular mechanisms (Gibson, 1994; Adams, 2002). Several indicators of either physico-chemical or biological quality of aquatic environments have been developed. Some are based on only one criterion as the Community Degradation Index (CDI; Ramm, 1988) and the Biological Health Index (BHI; Cooper et al., 1994), while others consist of a combination of several metrics, i.e. the

Indices of Biotic Integrity (IBI; Karr, 1981; Roset et al., 2007). In addition, some methods such as scope-for-growth (SFG) have long been used to good effect as an indication of anthropogenic stress in marine and estuarine areas (e.g. Widdows and Johnson, 1988). However, Navarro (1988) and Guerin and Stickle (1992) both indicate the way in which salinity stress, through natural freshwater inputs, reduces energetic budgets. Therefore as SFG will detect a reduction in physiological fitness due to salinity stress, there is the difficulty in using the technique for detecting and separating anthropogenic stress from natural stress. Other methods for detecting anthropogenic stress include those centered on the primary community structural variables (abundance, species richness and biomass) and derived community structural variables (such as diversity indices, abundance (A/S) and biomass (B/A) ratios, evenness indices) (Weisberg et al., 1997; McLusky and Elliott, 2004; Quintino et al., 2006).Moreover, following indices are currently used in many studies to report on the status of aquatic environments: IQBP (bacteriological and physicochemical index quality) used to assess the overall quality of the water. IBG (global biological index) and IBGN (normalized global biological index) assesses the health of the ecosystem of a river by the analysis of benthic macroinvertebrates or benthos (organisms living at the bottom of lakes and rivers, such as molluscs, insect larvae, worms, etc.); IBI (Index of biological integrity) used to identify and classify water pollution problems associated anthropogenic influences (Hartwell, 1997; Lydy et al., 2000; Dyer et al., 2000b; Kovacs et al., 2002; Porter and Janz, 2003). IPR (river fish index), IBMR (macrophytic biological index for rivers), IBD (biological index for diatomae), IOBS (oligochaetes index for Bio indication on muds) are the other indices used for the quality of ecosystems. Moreover, the European Water Framework Directive has provoked also great debate about the definition of ecological quality status (EcoQS) and the use of benthic bio-indicators and indices such as AMBI (Marine biotic index,), AFI (AZTI's fish index), BENTIX (bentic index), BQI (benthic quality index); and BOPA to determine the quality of transitional waters in Europe and along its coast (Borja et al., 2000; Simboura and Zenetos, 2002; Rosenberg et al., 2004; Dauvin and Ruellet, 2009; Pinto et al., 2009). Nevertheless, there are many multimetric or multivariate methods used for the benthic organisms such as ISS (Index of Size Spectra), BAT (Benthic assessment tool; Marques et al., 2009; Teixeira et al., 2009), NQI (Norwegian quality index, Borja et al., 2007; Josefson et al., 2009), M-AMBI (multivariate AMBI, Borja et al., 2004; Muxika et al., 2007), BEQI (Benthic ecosystem quality index; Van Hoey et al., 2007), BITS (Benthic index based on

taxonomic sufficiency; Mistri and Munari, 2008), and IQI (infaunal quality index; Prior et al., 2004) (Borja et al., 2011).

An increasing number of investigations have been focused on the search for organisms that serve as a means of monitoring biologically the impact of anthropogenic changes in these environments. For example, some general models were proposed connecting the occurrence of the faunas with the degree of eutrophication (e.g., Kitamori, 1984; Orive et al., 2002), whereas other more specific investigations have focused on the impact of selected consequences of this eutrophication in ecosystem functioning (i.e., hypoxia; Diaz and Rosenberg, 1995; Gray et al., 2002). Among macrofaunal organisms used in biomonitoring, both communities and individual species of bivalves (Cossa, 1995; Hiss et al., 1999), echinoderms (Fernández and Beiras, 2001; Beiras et al., 2003), sponges (Pérez et al., 2003), anemones (Harland et al., 1990), crustaceans (Rainbow and White, 1989; Clason et al., 2003) and fishes (Kress et al., 1998; Ueno et al., 2002) have usually been used as bioindicators or biomonitors (Rinderhagen et al., 2000) for this purpose. In addition, meiofaunal groups such as harpacticoid copepods (Lampadariou et al., 1997; Lee et al., 2001), turbellarians (Lee and Correa, 2005a), foraminifers (Alve, 1995; Yanko et al., 1999), diatoms (Cooper and Brush, 1991) and dinoflagellate cyst (Willard et al., 2003) have been used in biomonitoring for the evaluation of the effects of anthropogenic impacts. Ostracods are another meiofaunal group with increasing uses as biomonitors of stressed conditions in recent and quaternary environments (Malard et al., 1996; Mosslacher, 2000; Anadon et al., 2002; Boomer and Eisenhauer, 2002). Recent legislation worldwide requires suitable methods to assess anthropogenic impacts on marine ecosystems, using different elements of the system (Borja et al., 2008). Benthic macroinvertebrates, as one of these elements, have long been used to assess environmental impacts from human pressures (Littler and Murray, 1975; Pearson and Rosenberg, 1978; Dauer, 1993). Moreover, another important marine organisms, fish community structure has been also widely used to assess the effect of human impacts on aquatic ecosystems including water quality deterioration and habitat changes (Maret et al., 1997; Wolter et al., 2000; Angermeier and Davideanu, 2004; Pirhalla, 2004).The characteristics of fish communities including species diversity, total biomass and length frequency distribution can be considered as highly relevant endpoints since they reflect the health of the whole aquatic habitat, including habitat quality, food availability and water quality (Smith et al., 1999; Kovacs et al., 2002; MacDonald et al., 2002). A method frequently used for the assessment of fish community structure is the multi-metric index of biotic

integrity (IBI). The IBI was first introduced by Karr (1981) and is based on fish assemblage characteristics, such as species diversity, trophic composition and fish biomass. Since 1981 the IBI has been applied and adapted for different river systems in different countries (Kamdem- Toham and Teugels, 1999; Belpaire et al., 2000; Angermeier and Davideanu, 2004; Pirhalla, 2004). Another promising method to assess the quality of fish communities is the abundance/biomass comparison method (ABC-method). The ABC-method has been developed by Warwick (1986) as a method to assess the effect of water pollution on communities of marine macro-invertebrates. This method is based on the theoretical assumption that under 'stable' conditions interspecific competition will result in relatively low diversity equilibrium. Although the ABC-method has been primarily applied to macro benthos communities (Warwick et al., 1987; Beukema, 1988; Warwick and Clarke, 1994; Simboura et al., 1995; Harkantra and Rodrigues, 2004) it also has been used on birds (Meire and Dereu, 1990) and marine and freshwater fish communities (Coeck et al., 1993; Penczak and Kruk, 1999; Barletta et al., 2003).

Moreover, many other methods have been developed using morphometric, histological, biochemical and growth indices (Ferron and Legget, 1994). Comparison of several indices is advised and should indicate which is or which are the best indices for the estimation of marine organisms' growth or condition. A multi-biomarker approach to aquatic environmental monitoring allows the assessment of whole animal response to a range of anthropogenic disturbances. The use of short-terms (e.g. biomarkers) and mid and/or long terms (e.g. morphometric indices based on the length and weigth relationships, growth index, Fulton's condition factor K or biochemical indices such as RNA/DNA ratio, lipids storage index, TAG/ST ratio) can help to reflect the various environmental stressors on the aquatic ecosystems and marine organisms (Gilliers et al., 2004). For example, bioindicators along with biomarkers have been used to assess the health of aquatic systems. This approach uses responses of keystone (sentinel) aquatic organisms both as integrators of stress effects and as sensitive response (early warning) indicators of environmental health. Since many of the biomarkers are short-term indicators of long-term adverse effects, these data may permit intervention before irreversible detrimental effects become inevitable (Sanchez et al., 2007; Van der Oost et al., 2003).

Figure 3 presents the relationships between responses at different levels of biological organization as well as the relevance and time scales of these biomarker responses (Adams et al., 1989). Responses at each level provide information that helps to understand and interpret

the relationship between exposure and adverse effects of contamination. It is generally accepted that ecological relevance is inversely related to criteria like sensivity and specificity (De Zwart, 1995). Effects at a higher level of biological organization (population, community, etc.) have a high biological and toxicological relevance, but may be insensitive due to the presence of alternative pathways in an ecosystem.

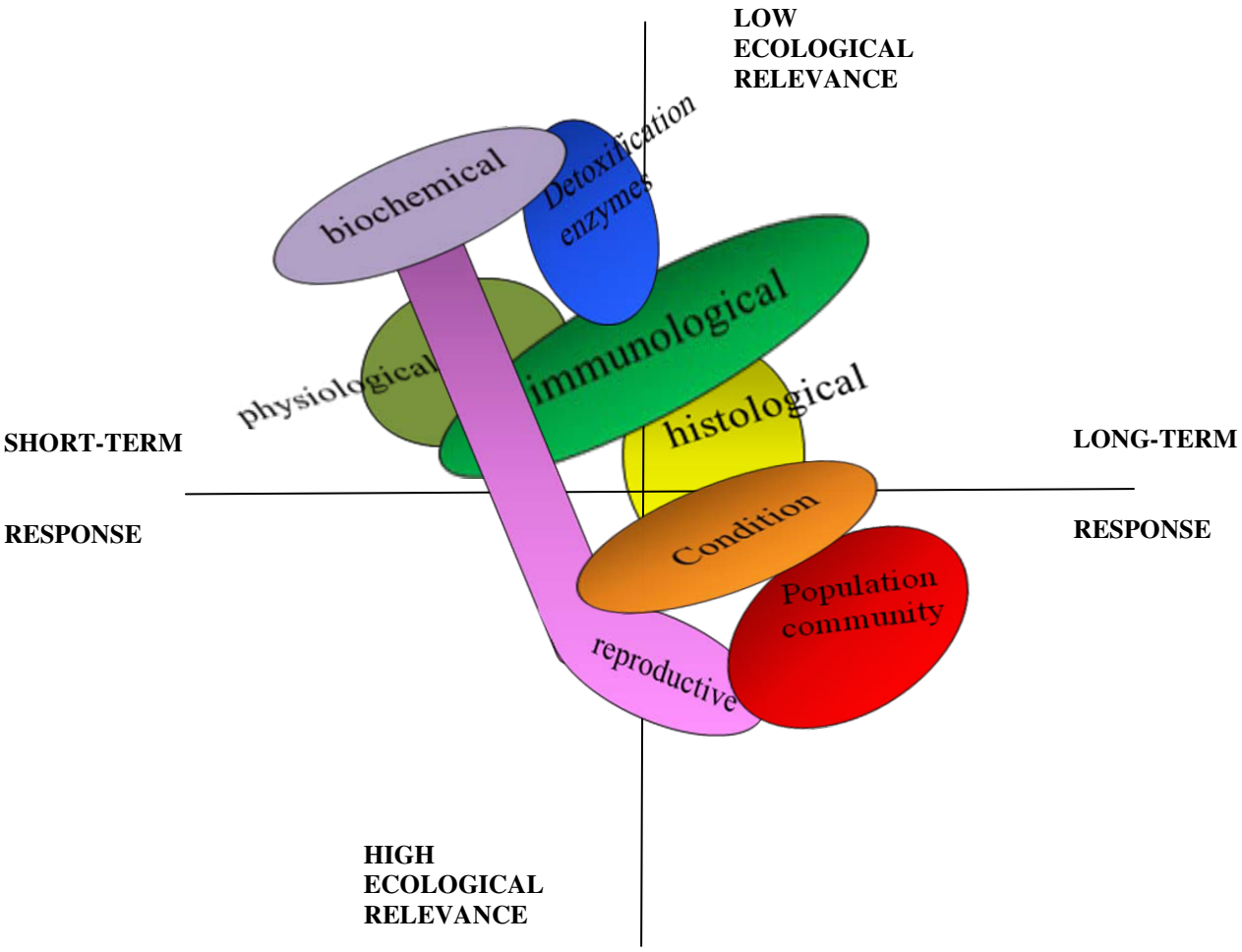



Figure 3. A theoretical visualization of the relationships between ecological relevance and time-scales of pollutant-induced biomarker responses (Adapted from Adams et al., 1989; Van der Oost et al., 2003)

In the last two decades, a variety of biomarkers has been successfully developed and adopted by various national and international monitoring programs in North America, Europe, Australia and New Zealand (e.g., International Council for the Exploration of the Sea (ICES), Convention for the Protection of the Marine Environment of the North East Atlantic (OSPAR Convention), National Oceanic and Atmospheric Administration (NOAA)), in order to: (a) identify exposure to certain chemicals (e.g., acetyl cholinesterase (AChE) for organophosphates, DNA adducts for genotoxicants, metallothionein for metals,and 7-ethoxyresorufin-O-deethylase (EROD) for PAHs or polychlorinated biplenyls (PCBs)), (b) monitor spatial and temporal changes in contaminant levels (e.g., body burden of metals and xenobiotics), (c) provide early warning to environmental deterioration (e.g., gonadosomatic index, conditioning factors, and pathological incidences) and (d) indicate the occurrence of adverse environmental consequences (e.g., imposex, population decline, and decrease in species diversity). However, it has long been suggested that molecular biomarkers should be used in conjunction with measurements of fitness to determine early responses of aquatic organisms (Huggett et al., 1992; Anderson et al., 1994; Cormier and Daniel, 1994; Malins and Ostrander, 1994; Depledge et al., 1995; Lagadic et al., 1998). Nonetheless, there are a modest number of studies demonstrating correlative relationships between biomarkers response and reduced fitness (e.g. growth and condition impairment, fecundity, pollution tolerance) of aquatic organisms exposed to toxicants *in situ* (Longwell and Hughes, 1980; Lesser et al., 2001) or in the laboratory (Sadinski et al., 1995).

In this context, over the last decade, a large number of single and multimetric indices have been developed, mainly in Europe and the USA (Díaz et al., 2004; Pinto et al., 2009). Much of this development has taken place within the European Water Framework Directive (WFD), trying to look for suitable methods to assess the benthic ecological status in marine and estuarine waters (Borja et al., 2009a). The European Water Framework Directive (Directive 2000/ 60/EC) states the need to achieve 'a good ecological status', by 2015, for all European water bodies, including transitional (estuaries) and coastal waters. Biological elements are especially important, in assessing such a status, e.g. phytoplankton, macroalgae, angiosperms, benthos and fish. A similar approach has been adopted by the new European Marine Strategy Directive (MSD; Directive 2008/56/EC), in assessing the environmental status within offshore waters (Borja, 2006), together with other legislation world-wide. (Uriarte and Borja, 2009). Another current approach, ''Driver, Pressure State, Impact, Response'' (DPSIR) approach (Borja et al., 2006) developed also to establish a framework for

the protection of different kinds of waters, including estuarine areas, in the context of the European Water Framework directive (Basset and Abbiati, 2004).

## I.4. Fish as bioindicator of aquatic habitats

According to Adams (1990) organisms like fish are continuously challenged or stressed by the normal demands of the aquatic environment and may be exposed to contaminants and to unfavorable environmental variables like temperatures, water velocities, sediment loads, dissolved oxygen concentrations, food availability and other variables. These factors can impose stress on their physiological systems.

Fish respond to chemicals and other stressors at intensity levels that are often far below those that can be detected by terrestrial animals. Fish are more sensitive to stressors than many other vertebrates because their physiological homeostasis is intimately bound to and dependent upon the water in the surrounding environment (Selye, 1973; Wendelaar Bonga, 1997). The representation of the pollutants effects on health curve illustrates different stress level on fish (Figure 4). Those stresses are characterized by different physiological conditions depending on the pollutant load. When the pollutant load is moderate, exposure causes a biological adjustment followed by a return to normal when the stress disappears. If the pollutant load is greater, other biochemical or physiological mechanisms involved in detoxification or excretion of pollutants tend to compensate the stress. When the load of pollutants exceeds the resilience of the body, it is the phase of non-compensation leading to pathologies and death (Burgeot et al., 1999).

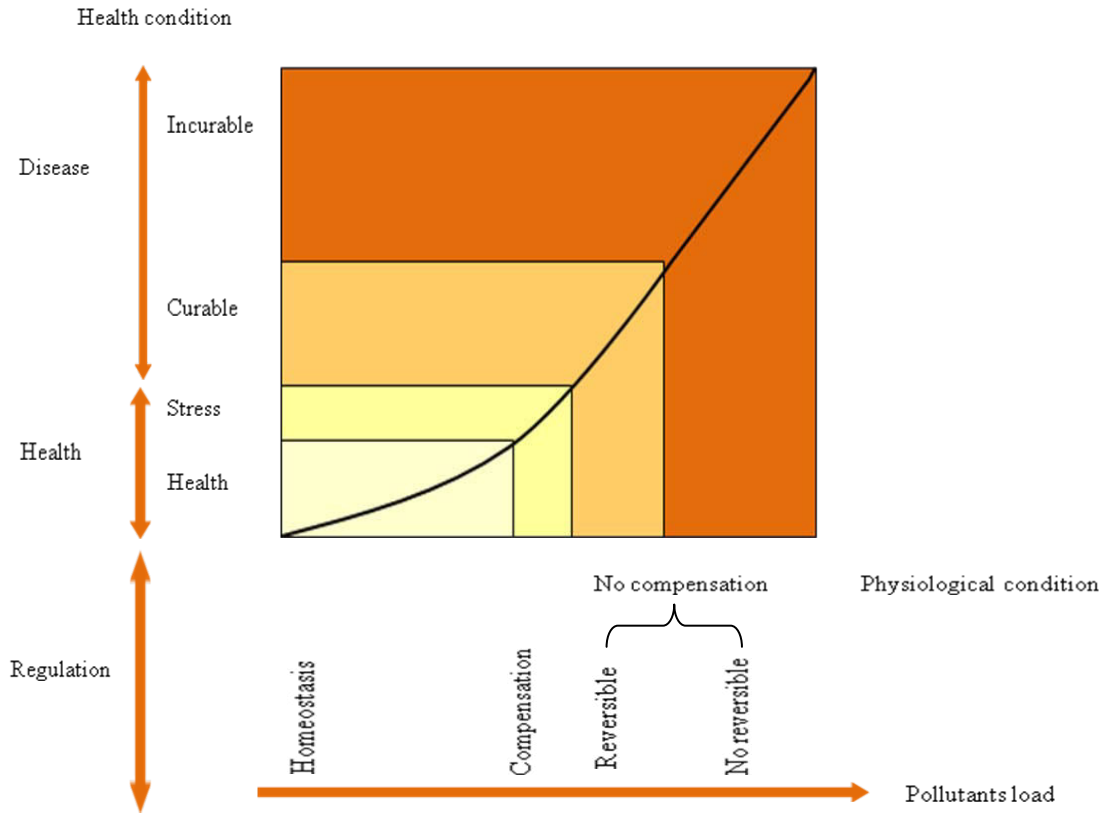



Figure 4. Presentation of different levels of the health condition of an organism depending on the pollutant load (Depledge, 1994)

Fish populations have both temporal and spatial sensivity to pollutants and other water quality parameters that can be measured instantaneously, both in the short-term and in the long-term. The sensitivity of fish to environmental stressors may vary at different stages in their life cycle prior to, or after entry into, the fish population (Landsberg et al., 1998). Fish are important members of aquatic communities are vulnerable to the effects of many human activities. The Fish's environment is a complex system with varying water quality (physical and chemical composition), current velocity, abundance and type of aquatic vegetation, light intensity and periodicity, temperature, food availability and social interaction.

Fish display close physiological relationships to their environment as ectothermic organisms. Thus, they are sensitive to environmental disruptions, and particularly to chemical stress. That is why other approaches such as analyses of fish biological responses to specific and multiple stressors have been extensively used to determine individual health and population status, and to assess habitat quality (Phelan et al., 2000; Alquezar et al., 2006; Fonseca et al., 2006; Costa et al., 2009a; Franco et al., 2010). In other words, to monitor the

quality of coastal and estuarine zones, the use of biological indicators that take into account their ecological function is becoming a widespread method (Basset and Abbiati, 2004; Coates et al., 2007). Indicators based on fish communities are recognized as useful tools to assess anthropogenic impacts on estuaries (Costa and Elliott, 1991; Deegan et al., 1997; Hughes et al., 1998; Whitfield and Elliott, 2002; Borja et al., 2004; Harrison and Whitfield, 2004; Harrison and Whitfield, 2006; Breine et al., 2007). For example, several measures of growth and condition of larvae and juvenile fish have been used to assess individual and population status as well as habitat quality (e.g. Yamashita et al., 2003; Gilliers et al., 2004; Fonseca et al., 2006; Amara et al., 2009). These measures comprise growth indices (RNA:DNA ratios, protein specific growth rate, otolith increments), morphometric indices (Fulton's K) and storage indices (lipid content) that relate to the individual ability to respond and interact with the environment at different time scales (Suthers, 1998). In previous studies, consistent differences in growth, condition indices (lipid storage) and abundance of 0-group sole among sites that present various degrees of contamination within the English Channel and the Bay of Biscay were shown: juvenile sole caught in nurseries located near harbour and close to, or within, polluted estuaries presented lower growth, lower condition indices and lower abundances (Gilliers et al., 2006; Amara et al., 2007). Sites highly impacted by anthropogenic disturbances in the form of contamination by heavy metals and organic contaminants were shown to provide low quality habitats for juvenile fishes (Whitfield and Elliott, 2002) with consequences on fish growth and survival and population renewal (Gibson, 1994). Because of their size, large estuaries are the most favourable to marine juveniles and potentially shelter greater diversity and abundance (Meng et al., 2002; Whitfield and Elliott, 2002). However, urban and industrial development in estuarine zones also leads to important losses of habitat for the juveniles (Lotze et al., 2006; Le Pape et al., 2007; Coleman et al., 2008). The quantity of available habitat for the juveniles is directly linked to the capacity of nursery areas (Gibson, 1994; Able et al., 1999) and the protection of these essential fish habitats is a key issue for fish population renewal and ecosystem management (Beck et al., 2001).

By linking metrics of good ecological status, based on fish assemblages and nursery function, to proxies of human disturbance, it allows development of indicators for monitoring networks, for the assessment of both water quality and ecological function of estuarine systems (Coates et al., 2007). Peterson et al. (2000) presented the need to take into account fish habitats and fish communities, and especially nursery ground, to prevent degradation in

ecological function of estuaries and fish population renewal. Whitfield and Elliott (2002) pointed out the interest of monitoring estuarine health using fish studies and Franco et al. (2008) emphasized the fact that the guild approach may be useful within the WFD for transitional waters, as it may provide valuable information on the ecological status of European transitional water bodies. In that WFD purpose, a large data set was created in France from fish samplings carried out within 13 estuaries presenting various degrees of human activity (Courrat et al., 2009). Moreover they showed that the nursery function is one of the main functions insured by North Sea and Atlantic European estuaries, as marine fishes are one of their main components in term of ecological guilds. Thus, the state of the nursery function of estuaries might be a good proxy for their broad ecological status, and especially because juvenile fish usually stay attached to their nursery site: they generally do not migrate between estuaries and are strongly habitat specific (Amara et al., 2007). Even if they may migrate between different nursery sites located within the same estuary (Vinagre et al., 2008a), they remain in an environment heavily influenced by estuarine water (Le Pape et al., 2003). Hence juvenile fishes that use estuaries as nursery grounds are likely to be impacted by anthropogenic disturbances. In the particular case of fish, the WFD specifies that they must be assessed in freshwaters and transitional waters (and not in coastal waters), taking into account species composition, abundance and the proportion of disturbance-sensitive species. In fact, the trends in one or more of the community attributes (such as composition, trophic structure, diversity, abundance or biomass) can be used to monitor the ecological functioning, and health, of an estuarine ecosystem (Moore et al., 1995; Whitfield and Elliott, 2002). As stated by Coates et al. (2007), most of the methods used to assess the ecological status, based upon fish, are derived from the metric-scoring system used in assessing the 'biotic integrity' of North American fish communities (Karr, 1981), e.g. the 'index of biotic integrity' (IBI). Derivations from this method have been used as a classification tool for fish quality assessment, world-wide (Deegan et al., 1997; Harrison et al., 2000; Gibson et al., 2000; Hughes et al., 2002; Whitfield and Elliott, 2002; Harrison and Whitfield, 2004, 2006); in recent times, it has served as basis for several methodologies applied under the WFD (Borja et al., 2004, 2009b; Breine et al., 2004, 2007; Coates et al., 2007), being some of them compared in Martinho et al. (2008). According to the WFD, biological element methodologies used to assess ecological status should respond to anthropogenic pressures, rather than to natural variability (Solimini et al., 2006). However, very few studies have focused upon the response of these fish assessment methods to human pressures (Harrison et al., 2000; Cabral et al.,

2001; Breine et al., 2007; Vasconcelos et al., 2007). In this case, the CEMAGREF team, Delpech et al. (2010) was reported an original methodology, multimetric fish-based index, based on a pressure-impact approach to characterize the ecological quality of French estuaries. This index of contamination, based on the chemical pollution affecting aquatic systems, was used as a proxy of anthropogenic pressure and it appeared particularly relevant to detect the contamination effects on fish communities in estuaries. It could help managers to take decisions in order to maintain or reach the good status required by the Water Framework Directive for 2015. Hence, there is a need to validate the proposed fish methodologies, against transitional water pressures, as has been undertaken for benthos (Borja et al., 2009a).

## I.5. Thesis Objectives and Organization

In this context, the global objective of this study is to estimate and understand the effects of environmental stressors, both natural and anthropogenic, on fish species, considering both *in situ* and experimental approaches (microcosm and mesocosm).

The thesis is structured into five chapters with the first chapter providing an overview on key topics, and introducing the current scientific knowledge on the subject.

Chapter II presents the materials used and methods followed throughout this study. It is divided into two parts. The first part describes the study area and sampling strategies with analysis applied in field study for the juveniles flounder. The second part stated the experimental studies on the contamination of sediment and algal blooms on juveniles of European sea bass.

Chapter III focuses on the effects of pollution on fish and divided into two sub-chapters. Chapter III.1. presents the work on the sensitivity of European flounder juveniles (*Platichtys flesus*) to chemical contamination in four estuarine habitats (Canche, Authie, Somme and Seine) and examined their utility as a monitoring tool for fish habitat quality. This chapter is also meant to improve our knowledge about the physiological performance and bioaccumulation of chemical contaminants on juvenile fish in different estuarine pollution gradient. Many questions to address this chapter have been raised: What is the impact of contaminants on growth and condition of juvenile flounder? To what extent do the juvenile flounder from different nursery areas bioaccumulate pollutants? Which nursery habitat is better for the physiological performance of juvenile flounder? How hydrological conditions or food availability affected on the flounder biological responses? Are fish biological responses better in clean nursery habitats than the heavily impacted estuary? Which

biological indicators are more sensitive and relevant to assess the quality of nursery habitat? All these issues will be addressed from indices measured in juvenile flounder in the study area.

Chapter III.2 exposes the results of European sea bass juvenile responses (*Dicentrarchus labrax*) related to physiological performance, biomarkers and immune systems in different scales of sediment contamination. This chapter should clarify how sediment chemical contamination could influence on fish fitness, more generally on fish health, experimentally, under controlled conditions (microcosm). This study is intended to publicize the potential usefulness of these indicators to examine pollutants stress in fish for habitat quality assessment and to reveal the value of biomarkers used for biomonitoring studies in natural environment. To address this aspect, different questions were asked: Are juvenile fish affected in different contaminated condition? How sediment contamination influence on their physiological performance? Are contaminants reduced fish fitness (physiological performance)? Which bioindicators are more sensitive and relevant to the effects of contamination? Is there any correlation between biomarkers and immunity (short term) and physiological performance (long term) responses?

Chapter IV. states about another environmental stress beside the contaminants is that the effects of the most recurrent two algal blooms in the northwest European shelf seas, *Phaeocystis globosa* and *Pseudo-nitzschia pseudodelicatissima* that can be directly or indirectly deleterious for human and marine organisms such as shellfish and fish. This chapter divided into two sub-chapters. This chapter is attended generally to assess about the ichthyotoxicity of these algal blooms on the growth and survival consequences for highly mobile animals, such as fish juveniles that use inshore areas for all or part of their life cycle under controlled conditions (mesocosm). Chapter IV.1. exposes about the effects of exudates and transparent exopolymeric particles (TEP) produced from *P. globosa* bloom senescence on the physiological performance and mortality of European sea bass juveniles. To address this aspect, different questions were asked: Are sea bass juveniles affected by TEP or foam form of *Phaeocystis globosa*? Which are the physiological responses of fish juvenile against this contamination? TEP particles can be a source of food for juvenile fish? Is there any evident mortality or abnormalities recorded due to this contamination? Chapter IV.2. presents the work of the impact of *Pseudo-nitzschia pseudodelicatissima* on the growth and condition of European sea bass juveniles. In this chapter, following questions are asked: Does *Pseudo-*

*nitzschia sp.* affect on the fitness of juvenile fish? Which are the physiological responses of juvenile fish against this contamination?

Finally, Chapter V. is the general conclusion. It summarizes the overall results of the thesis and outlines the main findings related to the environmental stressors on fish either *in situ* and experimentally. The perspectives are described also in this chapter.

# CHAPTER II

# METHODOLOGY

## II.1. *In situ* approach

The French coast of the English Channel composes our study area (50° 06' N, 1° 30' E) (Figure 5). This zone extends from the Cape Gris nez, located between Calais and Boulogne at north, to the Cap of La Hague in the south. The Eastern English Channel is an arm of the Atlantic Ocean that separates Great Britain from northern France, and joins the North Sea to the Atlantic. It is about 560 km long and varies in width from 240 km at its widest, to only 34 km in the strait of Dover. It constitutes a zone of transit water masses between the Atlantic Ocean and the North Sea. It is the smallest of the shallow seas around the continental shelf of Europe, covering an area of some 75,000 $km^2$. The channel is relatively shallow, with an average depth of about 120 meters at its widest part, reducing to about 45 meters between Dover and Calais. This area is characterized by semi-diurnal tidal regimes, strong hydrodynamism and sand-gravel substratum. The English Channel is known as the world's busiest seaway. Cross-channel passenger crossings and trading has been a significant factor for societies on both sides of the Channel from prehistoric times, and a number of important seaports and ferry locations have developed in both England (Dover, Southampton, Plymouth, Weymouth, Portsmouth, Poole, Newhaven) and France (Calais, Caen (Ouistreham), Dieppe, Le Havre, Cherbourg-Octeville, Roscoff, Saint Malo) (James et al., 2007).

The coast of the eastern part of the English Channel in North France is bordered by several important megatidal estuaries. Several authors have emphasized the importance of estuaries for marine fisheries by demonstrating that a large part of the landings around the world is made up of species that spend part of their life in estuarine waters (Pauly, 1988; Lamberth and Turpie, 2003). All of them have evolved quickly at the ecological scale, due to natural influences but also because of human influences. Compared to marine or freshwater systems, estuaries are abiotically variable and therefore may be rigorous and stressful habitats. Species that inhabit estuaries must be able to tolerate or avoid wide range of salinity, temperature, dissolved oxygen and high level of turbidity. Moreover, these estuaries perform a crucial role in the population dynamic of many invertebrate and fish species. They provide a migratory route for anadromous and catadromous fish species and function as vital nursery grounds for numerous commercially important fish species, including many juveniles (Claridge et al., 1986; McLusky and Elliott, 2004).

In this study, four important nursery grounds (Canche, Authie, Somme and Seine estuaries) were investigated in terms of different anthropogenic activities. These sites are

characterized by sandy-mud substratum, a semi-diurnal megatidal regime (tidal range ~ 5-8 m) and are influenced hydrodynamically by the spring tides (> 8-10 m in magnitude). The water circulation is mainly dependant on the tides and on a small freshwater input. The three small estuaries of the area, Canche, Authie and Somme, undergo limited human activities and can be considered as clean or little impacted systems (low domestic, agricultural and industrial effluents) (Amara et al., 2007). In contrary, the Seine estuary was chosen in relation to the high degree of contamination compared the other estuaries. This estuary is heavily impacted by manmade modifications and can be considered as one of the most contaminated in Europe (Chiffoleau, 2001; Dauvin, 2008).

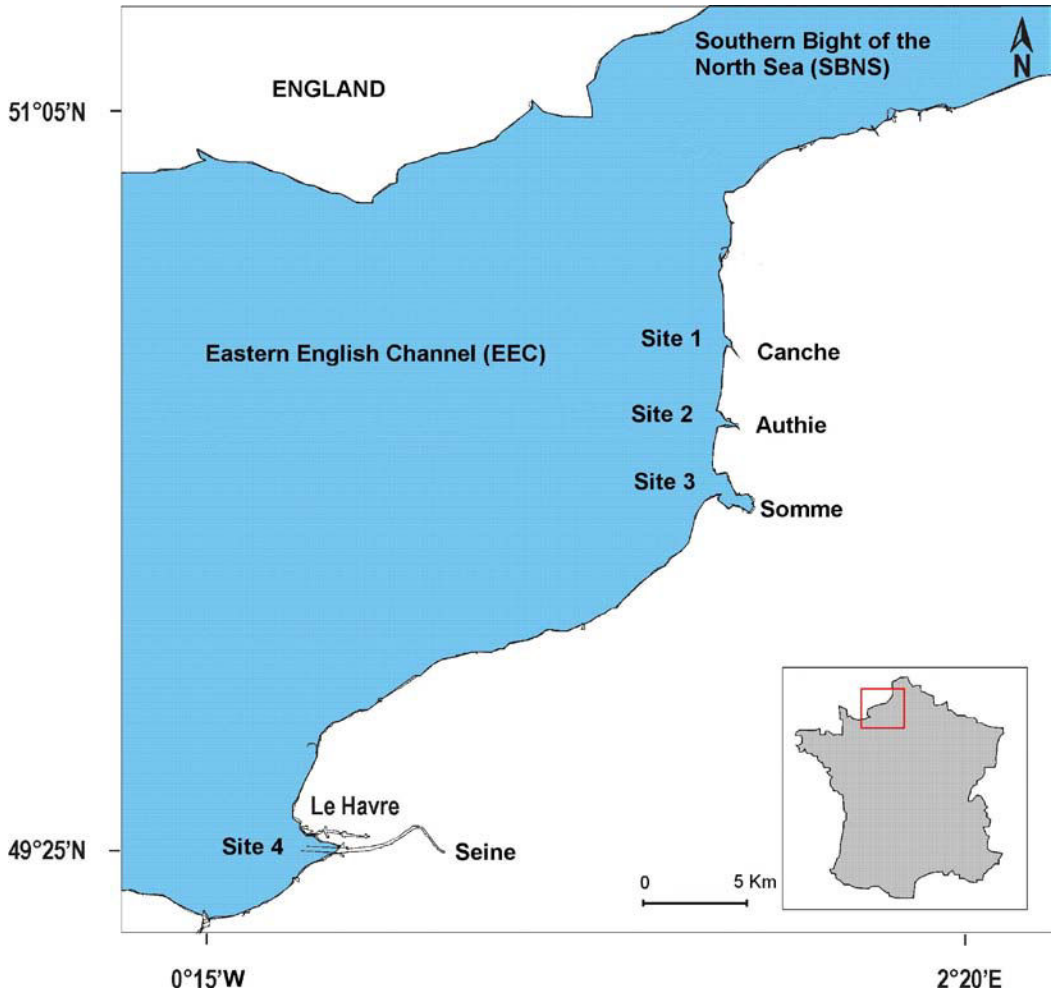



Figure 5. Location of study areas

### II.1.1. Canche, Authie, Somme and Seine estuaries

#### The Canche estuary

The Canche estuary is one of the rivers that flow from the plateau of the southern Boulonnais and Picardy, into the English Channel. It is located in the department of Pas-de-Calais and is bordered by the town of Etaples in north and Touquet in the south. The Canche is a small estuary approximately 10 km in length and 1.5 km in width at its mouth, 7.8 $km^2$ of surface and 6.9 m tidal range with a mean annual average of 11 $m^3$ $s^{-1}$ freshwater inputs. Forming an alluvial valley, the Canche is a verdant landscape of calm waters, marshes, meadows and small woods. The gentle gradient, averaging 1.5%, gives the river a meandering course. With sandbars and spits, the estuary of the Canche is typical of the estuaries of this region of France. The coastal dunes, marshes and valley are home to 485 different plants and a diverse range of wildlife. In terms of ichthyology, the Canche estuary was mostly used as temporary habitat by fish, as feeding or nursery grounds. Numerous individuals caught in this estuary were juveniles of euryhaline marine fish species (Amara et al., 2007; Selleslagh and Amara, 2008a).

#### The Authie estuary

Authie estuary is a macrotidal system (maximum tidal range of 8.5 m at its mouth) located in the northern part of France, at the border between the departments of Pas-de-Calais and the Somme and it is localised between Berck sur mer in the north and Fort-Mahon in the south. The mean annual discharge of the Authie estuary is 10 $m^3$ $s^{-1}$, and the estuary has a 985 $km^2$ catchment area and 12.8 $km^2$ of surface. The Authie, as a small estuary, is approximately 12 km in length and 1.5 km in width. This estuarine system is rapidly filling with silting, but a chief feature is the penetration of a substantial sand fraction originating from the English Channel. Morphologically, the Authie consists of a bay protected by a sand bar (located in subtidal to supratidal domains) at its mouth, which shelters the estuary from storm swells. The principal hydrodynamic feature is the rapid filling of the bay by the tide: during low tide, most of the estuary, except the main channel, is sub-aerially exposed, and during the flood period there is significant resuspension of fine sediment. The Authie estuary is considered to be a relatively “natural estuary”, compared with other local systems, although some polders

have been constructed, inducing a seaward salt marsh progression and increased sedimentation (Anthony and Dobroniak, 2000).

**The Somme estuary**

The Somme estuary, located between Le Crotoy at north and Saint Valéry at the south, is found within the region of Picardie. It is a large megatidal ecosystem of the eastern English Channel (France) with an intertidal area (excluding salt marshes and channels) of 42.5 $km^2$ occupied by seven distinct biosedimentary facies. It is characterized by a rather low fresh water input (35 $m^3$ $s^{-1}$) and strong hydrodynamic processes. Spring tides reach a height of 9.8 m and the salinity on mud flats rarely drops below 25 psu. The Somme has a 6550 $km^2$ catchment area. It is approximately 14 km in length because of its delimitation the upper estuarine part by the presence of a dam, but extends on approximately 6 km in width at its mouth. The Somme basin is dominated by intensive cereal agriculture, which forms the major part of the catchment area. The main socio-economic activities consist of traditional practices such as hunting, cockle and inshore fishing and harvesting of vegetable products. In the surrounding polders, traditional agriculture is surviving. For the past few decades, tourism has developed on a large scale and has helped reorganize entirely the local economy. In order to manage the environment in a sustainable way, local authorities have installed a management body, called ‘’Syndicat Mixte pour l’Aménagement de la Côte Picarde’’ (SMACOPI). The syndicate claims restoring and rehabilitating estuarine features as well as opening new facilities to tourists in the area. The population density is relatively low (100 inhab / $km^2$), with only three large urban centers. These are settled in the upper part (Saint- Quentin 60,000 inhab), in the middle part (Amiens 160,000 inhab), and along the downstream canalized part of the drainage network (Abbeville 30,000 inhab) (Cabioch and Glaçon, 1975; Amara et al., 2007).

**The Seine estuary**

The Seine estuary is one of the main estuaries of the North Western European continental shelf, providing the most significant fluvial input into the English Channel. Its geographic zone of influence runs from just upstream of the Poses dam – some 160 km upstream Le Havre, at the limit of the tidal penetration into the estuary – to the eastern part of

the Bay of Seine. This zone can be divided into three sections: the fluvial, or upstream, estuary from Poses to Tancarville Bridge; the middle estuary between Tancarville Bridge and Honfleur; and the marine, or downstream, estuary which opens into the Channel (Figure 6). The basin of Seine river (78 650 $km^2$) corresponds to 25% of French agriculture, 25 – 30% of French industrial activity, and 23% of French population on only 12% of the French territory.

97.5% of the basin, with a remarkably homogeneous geology, is covered with sedimentary rocks, including 78.2% of various carbonate rocks, such as chalk and limestone. It is characterized by a low relief, pluvial oceanic climate, (700 mm of rainfall a year), high water stage in winter and low water stage in summer. The freshwater flow of the river at Poses is relatively higher (480 $m^3$ $s^{-1}$ on average over the last 30 years), with high water volumes over 2220 $m^3$ $s^{-1}$ (autumn/winter) and low water flow under 100 $m^3$ $s^{-1}$ (at the end of summer in September). The Seine estuary can be classified as a macrotidal estuary (3 m tidal range at neap tides, 7.5 m tidal range at spring tides, based on data for Le Havre city) and is characterized by a high turbidity zone. This turbidity maximum is dynamic, but is typically found between Tancarville (9 km upstream of the Pont de Normandie) and Le Havre. Like most rivers in the world, human impacts and engineering have greatly altered the natural system of the Seine River, whose lower part was regarded as one of the most contaminated rivers in the world before 1970. The annual high and low water stages have been modified from their natural extremes by upstream reservoirs that modulate the river flow. Dams and locks have been installed and the river dredged to aid navigation (Durou et al., 2007; Rochette et al., 2010). Currently, Seine estuary is considered as one of the most polluted estuaries in Europe with respect to potentially harmful elements, receiving effluents from the upstream Paris urban area, and also local inputs from the heavily-industrialized Rouen and Le Havre regions (Miramand et al., 2001).

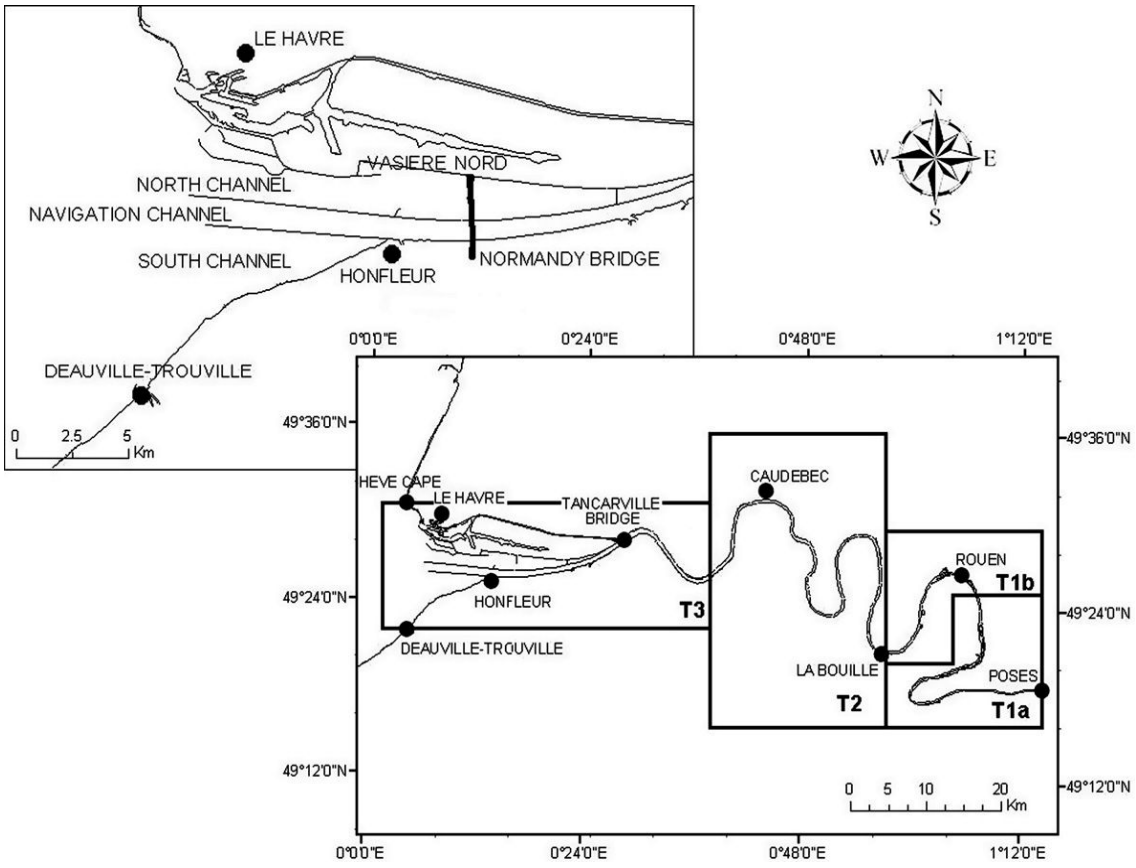



Figure 6. Detailed maps of the Seine Estuary, showing the various transitional water masses –T1a and T1b are freshwater tidal, T2 is saline tidal, T3 is the open estuary (Dauvin, 2007)

### II.1.2. Choice of European flounder (*Platichthys flesus,* L., 1758) as a biological model

The shallow marine coastal zones of the eastern channel and southern bight of the North Sea provide important nursery habitats for juvenile flatfish (Riou et al. 2001). First of all, young fish utilize estuaries and near-shore marine areas as nursery in order to benefit from the availability of food and perhaps also to gain protection from predators (McLusky and Elliot, 2004). In addition, juveniles in estuarine nursery areas tolerate and overcome some of the occurring environmental constraints (Vasconcelos et al., 2009). Hence, they can settle down in estuaries as their nursery grounds during an important part of their life and can reflect the environmental conditions changing. Finally, juvenile's fish, as free-living and fast-developing organisms, are also highly susceptible to pollutants in the environment. Investigating, *in situ* and experimentally, how environmental disturbances affect the quality of

fish juveniles is a major importance to understand their consequences on the population renewal of marine species (Rochette et al., 2010).

The European flounder (*Platichthys flesus*) is a flatfish of European coastal waters from the White Sea in North to the Mediterranean and the Black Sea in South. *P. flesus* was chosen as a suitable species *in situ* study for several reasons (Figure 7). Firstly, it widely distributed in marine and brackish habitats throughout Europe, especially, along the French coast of the Eastern English Channel (EEC) in low salinities during the first months following their settlement. Juveniles of this species concentrate in estuaries and are one of the most important components of the demersal fish assemblage in European estuarine waters. Secondly, it is common in both polluted and clean estuaries; and it prefers fine-grained to sandy sediments. As with many other flatfish, flounder migrate to deeper waters (20-60 m) during the winter months to spawn but return to shallow water (2-15 m) in the same estuary during the summer period. Finally, the European Flounder is commonly used in biomarker studies previously and also for environmental monitoring and toxicology studies in France and northern European waters. For example, this species has been adopted by the OSPAR Joint Assessment and Monitoring Programme as the sentinel species for biological effects monitoring in inshore/estuarine waters of the OSPAR maritime area. Therefore, they are more sensitive to the effects of pollution and other types of habitat degradation, since they feed on benthic organisms and live in close association with the bottom sediments, where most of the chemicals introduced into aquatic environments by human activities accumulate. (Sulaiman et al., 1991; Minier et al., 2000; Adams, 2002 ; Marchand et al., 2004; Kirby et al., 2004; Williams et al., 2006; Selleslagh and Amara, 2008a).

Figure 7. Juvenile of European flounder (*Platichthys flesus)*

### II.1.3. Sampling strategies

Juvenile flounders were collected in spring (late May - June) 2008 in the oligo- and meso- haline zones (salinity between 0 and 18) (Figure 8). Sampling was performed using a 1.5 m beam trawl at high tide, with one tickler chain and 5 mm mesh size in the cod end, towed by a zodiac against the current at 2 knots for 15 min (Figure 9). The depth of flounders sampling ranged between 1.8 - 5.3 m in the four estuaries. Immediately after sampling, 0-group flounders were sorted alive on board and individually reserved in polyethylene bags in ice box in the field, then transported to the laboratory and stored at -20°C prior to analysis.

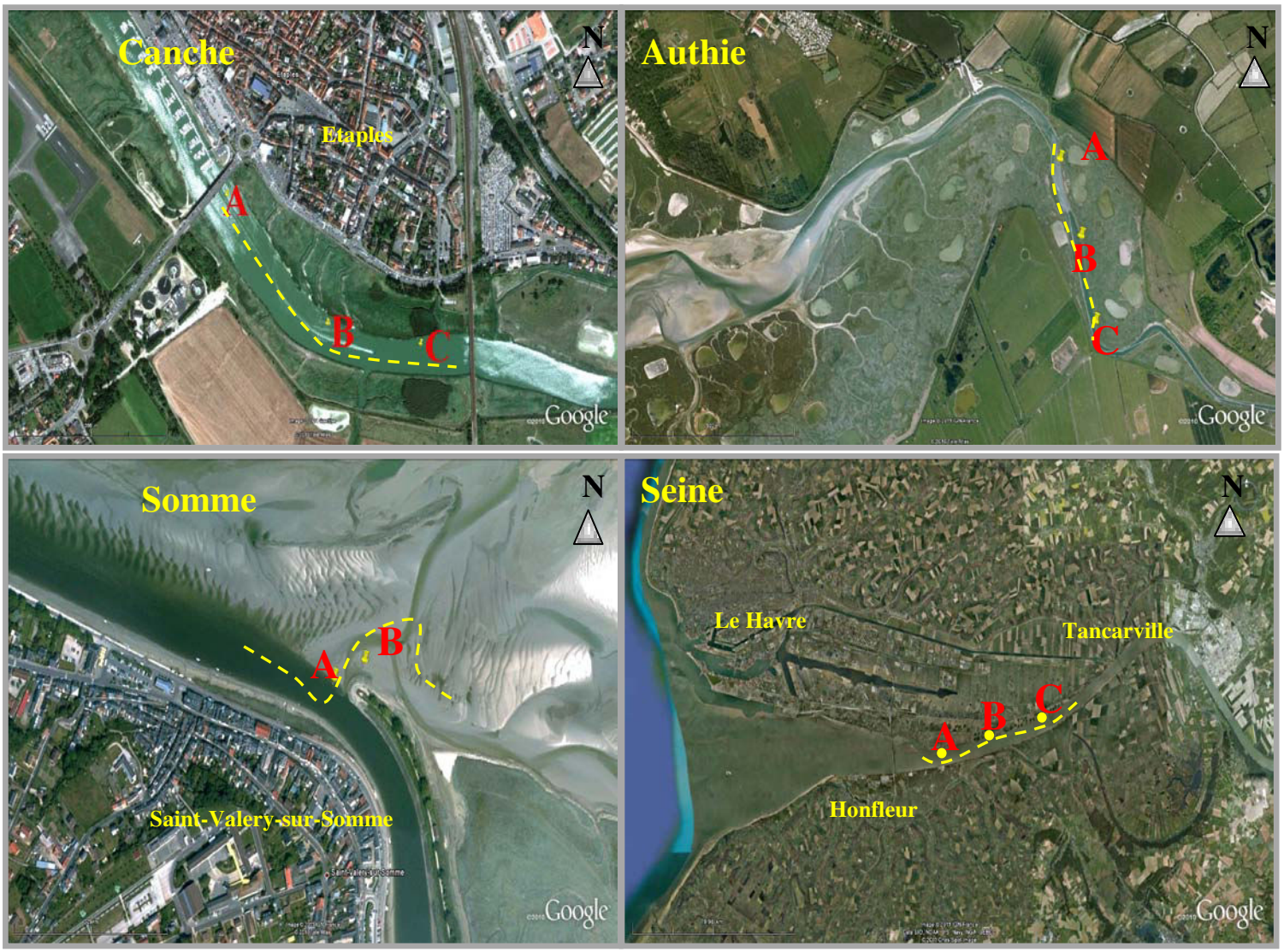



Figure 8. Sampling stations of juvenile flounders in the four estuaries (Canche, Authie, Somme and Seine) (A,B,C: sediment sampling stations, dotted line: trawling zone)

Figure 9. Beam trawl and zodiac used for fish sampling in the estuaries

### II.1.4. Measurement of environmental parameters

Fish species are closely related to its environment and reflects both environmental conditions and integral factors (Liasko et al., 2010). As estuaries are important nursery grounds, it has been reported that such environmental influences are most likely to affect juveniles during their estuarine residency (Attrill and Power, 2002). In order to understand this relationship between fish juveniles and environmental factors, some abiotic and biotic variables were integrated in this study.

#### II.1.4.1. Physicochemical parameters

Prior the fish sampling, water physicochemical parameters (temperature (°C), salinity, oxygen (mg/l), conductivity (µS/cm) and pH using a Hanna HI 9828 multiprobes; turbidity (NTU) using a waterproof turbidimeter (Eutech instruments, TN-100) and depth (m) using a Garmin Fishfinder 250 sounder) were measured in each station of estuaries.

#### II.1.4.2. Sediment sampling

Some additional environmental parameters such as total macrobenthos abundance as a trophic indicator of the environment, granulometry, organic matter in sediment were measured during June 2008. Three stations were chosen (except for Somme with two stations) for each parameter to collect sediment samples using a small type of Van Veen grab (250 $cm^2$ sampling area to a sediment depth of ~ 10 cm) (Figure 10) in all estuaries and aliquots of

sediment were used: one for macrobenthos analysis, one for sediment particle size analysis and the third for organic matter (OM) content. Samples were preserved in plastic bags and transported to laboratory. Superficial sediments were also collected at low tide and kept at – 20 °C until further treatment and analysis of chemical contaminants (metals, PAHs and PCBs).

Figure 10. A type of Van Veen grab used for the macrobenthos and sediment sampling and preservation

### II.1.4.3. Sediment analysis

#### II.1.4.3.1. Macrobenthos

Macrobenthos samples were washed and sieved through a 1 mm mesh in the laboratory. Organisms retained by the sieve were preserved in 5% formaldehyde buffer mixed with Rose Bengal until they could be identified to species level and the unidentified ones were classified in the generic level or up to the lowest taxonomic level. Those organisms were counted using a binocular microscope and their abundances (individuals.$m^{-2}$) and species occurrence (%) were calculated for each estuary. In addition, the dry weight (DW) of each taxon was determined after oven-drying at 80~ for 48 h (Crisp 1971) and the ash weight by further heating to 550~ for 3 h (Mason et al. 1983). AFDW (Ash Free Dry Weight, g.$m^{-2}$) biomass values were then measured as the difference between these two values..However, for the Seine estuary, we observed any macrobenthos abundances. Therefore, we used the database called Macrobenthos of the Bay and Estuary of Seine (MABES); available via the

data administrator of the GIP Seine Aval: nbacq@seine-aval.fr). From this database of the year 2008, we choose 3 stations located near the flounder sampling.

### II.1.4.3.2. Granulometry

Grain size were determined in the upper 15 cm layer of an humid aliquot using a laser Beckman-Coulter LS230 size analyzer (Range 0,4 µm - 2mm) (Figure 11). Granulometric classes are given in volume percentage using this classification: clay (<4 µm), fine silt (4 - 20 µm), coarse silt (20 – 50 µm), fine sand (50 - 200 µm), medium sand (200 - 500 µm) and coarse sand (500 - 2000 µm).

Figure 11. Laser particle size analyser

### II.1.4.3.3. Organic Matter

For the measurement of total organic matter (TOC, $mg.g^{-1}$) within the sediment, samples (~ 2 g) were dried at 60°C for 72h and subsequently burned at 500°C for 6h and was calculated by the difference between total dry-weight and ash-weight of sediments (Luczak et al., 1997). In addition, the total organic carbon, (TOC) and total organic nitrogen (TON) contents were determined using a CHNS analyzer (NA 2100, CE instruments) (Figure 12).

Figure 12. CHNS analyser

### II.1.5. Feeding analysis

One hundred eighty juvenile of flounders captured during May-June 2008 in four estuaries (Canche, Authie, Somme and Seine) were dissected, and their gut (stomach + intestine) content removed and fixed immediately after capture in 90% alcohol for later analysis. Only contents of the foregut were examined, because nearly all items in the mid- and hindguts were digested beyond recognition. The foregut of each flounder was split open, the contents placed in a Petri dish containing a small amount of water, distributed as evenly as possible, and viewed under a stereomicroscope of type Leica Wild M10 (magnifications until 80X). Gut contents were categorized as copepods, amphipods, isopods, ostracods, polychaetes, nematodes, molluscs, insects' larvae, teleostei, egg or terrestrial plant detritus. The main prey categories were identified and counted to the lowest possible taxonomic level. The mean prey number (the mean of the each examined food category) and the percentage of empty gut (% empty stomach = number of empty stomach / number of total examined stomach) were calculated for each estuary. Juvenile of flounders were refrozen immediately after dissection.

## II.2. Experimental approaches

It has been carried out three experimental studies with different contamination conditions (heavy metals in sediment and algal bloom). These experiences were performed on juvneile stages of European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758) to detect and to assess the effects of acute and/or chronic contamination on physical fitness of fish. The results of these experiences will be compared with the *in situ* study to evaluate different environmental stressors on juveniles' fish.

These experiments were conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. The University of Littoral Côte d'Opale is authorized to conduct experimentation on animals in its capacity as a certified establishment; according to the administrative order N° B62-160-2.

### II.2.1. Choice of European sea bass (*Dicentrarchus labrax,* L., 1758) as a biological model

European Sea bass is a euryhaline key species in estuarine and coastal environments and a valuable commercial aquaculture species, economically and ecologically, in the Mediterranean countries (Figure 13). Juveniles of this species spend most of their first year of life in estuaries or lagoons and can survive in a wide variety of temperature and salinity (usually lower than in the open sea: 10–20‰ vs. 30–40‰) levels in the open sea, the brackish river deltas, the estuaries and the Mediterranean lagoons (Saillant et al., 2003; Varsamos et al., 2006). In addition, there is considerable knowledge about its physiology and biology and protocols for maintaining this species in captivity in good condition are well established. With the technical development of artificial spawning and larval rearing over the past two decades in Greece, *D. labrax* has proved to be a suitable species for commercial fish culture. In addition, knowledge of the pattern of early larval development and the effects of environmental parameters on reproduction, particularly in reared fish, is important as it facilitates aquaculture research and fish resources management. However, difficulties mainly in the early stages of its cultivation relating to skeletal deformities, feeding and rearing conditions restricted the number of available fish for further development (Conides and Glamuzina, 2001; Hatziathanasiou et al., 2002 ; Conides and Glamuzina, 2006; Moreira et al., 2008). This species has, for those reasons received an important scientific interest over the last 35 years, but aquaculture-related stress appears to have major negative impacts on the culture of this fish. Finally, *D. labrax* is commonly used in biomarker studies previously (Sabourault et al., 2001; Varò et al., 2003; Bado-Nilles et al., 2009; Tovar-Ramírez et al., 2010), environmental monitoring and toxicology studies and stress responses to environmental parameters on the early life of development or juveniles (Lemaire-Gony et al., 1995; Cattani et el., 1996; Romeo et al., 2000; Giari et al., 2007; Faucher et al., 2008).

Figure 13. Juvenile of European sea bass (*Dicentrarchus labrax*)

### II.2.2. Microcosm experience on sea bass juveniles (*Dicentrarchus labrax*, L., 1758) exposed to estuary sediment contamination

This experience was performed about the sublethal effects of chemical contamination and physiological performances of European sea bass to different level of contaminated sediment. Juveniles of sea bass from Aquanord marine hatchery (Graveline, France) (genetically homogenous) were transported to the laboratory in oxygenated balloons in the same temperature of sea water where they have been cultured in the hatchery. Juvenile fish has been chosen randomly but regarding the same range of size. In the laboratory of Marine station (Wimereux), juveniles of European Sea bass (< 1 years old, ~100 days) (n=115 fish/tank) were acclimatised during two weeks before the experiment in two of 160 L (100*40*40 cm) aquaria supplied with closed seawater circuit and the water was aerated with air pumps and filtered with external filter (900l/h) (JBL, Cristal profi e900). One-third of the seawater in each tank was renewed manually every 2 days. The photoperiod was maintained at 10L:14D in a thermostated room (15 ± 1 °C) and water temperature in tanks was kept constant at 14 ± 1°C as in the hatchery condition for juveniles of sea bass during the experiment. Fish were fed 1% of the mean body weight once a day with commercial dry pellets (Biomar S.A., Bio-Optimal Start N$^{o}$ 1.5) throughout the experiment including acclimation and exposure time. Water parameters such as temperature (°C), salinity, oxygen (mg/l), pH using a Hanna HI 9828 multiprobes and turbidity (NTU) using a waterproof turbidimeter (Eutech instruments, TN-100) were measured 2 times every day (at 10:00-17:00). Following the acclimatization, sea bass juveniles were anaesthetised (0.32 ml / l of 2-phenoxyethanol: ~3-4 min anaesthesia time, ~30sec recovery time), weighed (near to 10 mg), measured for total length (near to 0.1 mm), and individually marked (Visual Implant Tag, 1.2 mm x 2.7 mm, Northwest Marine Technology) before exposure. Afterwards, thirteen fish were sampled at the beginning of the experiment as reference ($t_0$). Fish were then rapidly transferred to the thermostated and aerated room (15 ± 1 °C) at the Marine station of Wimereux into duplicate aquariums of 30 l (20*30*50 cm) randomly where 5 L was filled with sediment and 25 l with seawater aerated with air pumps (14 ± 1°C). For each condition, sediment let settled during 2 days to avoid release of suspended particles in the water. Three contaminated (Seine estuary: Normandie Bridge, Caudebec and Rouen), one less impacted system (Canche estuary) and one reference (Wimereux) sediment was collected with a plastic spatula from surface up to a depth of ~10 cm during low tide and stored in polyethylene bags prior to the exposure experiment. GPS coordinates and sampling dates for each sampling site

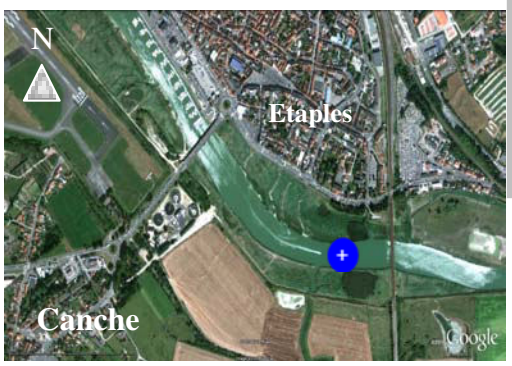
were recorded: Normandie Bridge (49°26' 44.64" N, 0°15' 01.94 E), Caudebec (49°31' 25.84" N, 0°44' 14.32 E) and Rouen (49°23' 48.81" N, 1°01' 05,82 E )) (26 January 2010), Canche estuary (50°30' 35.57" N, 1°38' 30.38 E) (3 February 2010) and Wimereux as control station (50°46' 00.87" N, 1°36' 04.82 E) (4 March 2010). About 10 L of sediment of each condition was transported to the laboratory. The fractions samples dedicated to chemical analysis was frozen and stored at – 20 °C in order to determine granulometry, organic matter, metals and its bioavailability, PAHs (EPA's 16 priority PAHs) and PCBs (7 congeners) contents . Beside this, it has been analyzed also heavy metals in sea bass juvenile's gills. The remainder of the sediment was stored at -4°C during few days until the exposure experiment.

During the exposure time, one-third of the seawater in each tank was renewed manually every day, the photoperiod was maintained at 10L:14D, fish were fed 1% of the mean body weight once a day and water parameters were measured for each aquarium. In order to monitor growth and physiological performances of juveniles, 10 individuals per aquarium were removed at the end of experiment ($t_{21}$) and rapidly transferred to the laboratory (within 2 h), anaesthetised, identified (tag), weighed, and measured for total length. For biomarker analyses after one week of exposure ($t_7$) livers of 10 fish per aquarium (20 per condition) were sampled, frozen in nitrogen liquid and stored at -80°C. Muscles and gills on $t_{21}$ were stored at −20 °C, respectively, for biochemical and metal bioaccumulation analysis. Daily observations were carried out every morning before the first food supply to assess fish mortality. In order to demonstrate experimentally sublethal effects of contaminated sediment on the physiological performances of sea bass juveniles, it was examined the correlative relationships between short term responses analyzing biomarkers (detoxification enzymes: EROD, GST and oxidative stress enzymes: catalase (CAT)) and long term responses measuring growth rate in length and weight, RNA-DNA ratio and relative condition index (Fulton's K). In order to determine the impact of contaminated sediment on the immune system of fish juveniles, two immunocompetent organs: thymus and spleen were sampled for each condition at the end of experiment and stored at -80 °C before analysis. This party were analysed by Frauke Seeman, PhD student in LEMA laboratory (Laboratoire d'Ecotoxicologie-Milieux Aquatiques) of the University of Le Havre (France). In spleen, the number of melanomacrophage centers per area ($MMC/\mu m^2*10^{-6}$) (10 random cuts per 5 fish of each treatment) was investigated as the innate immune responses. The accumulation of melanomacrophage centers (MMC) is used as a general marker for stress in fishes as well as it is an indicator for enhanced phagocytoses of particles. In thymus, it is analyzed the adaptive

(comparison of mature and immature T-cells in cortex and medulla, volume of thymus, cortex and medulla, cortex / medulla ratio) immune responses for the control and the most contaminated (Rouen) condition. Both thymi per fish (n=5) were examined and a section every $100^{th}$ µm was studied.

## First experiment Summary

Sediment collection zones ( ) : Wimereux (reference site), Canche (less impacted site) and Seine (impacted sites) estuaries

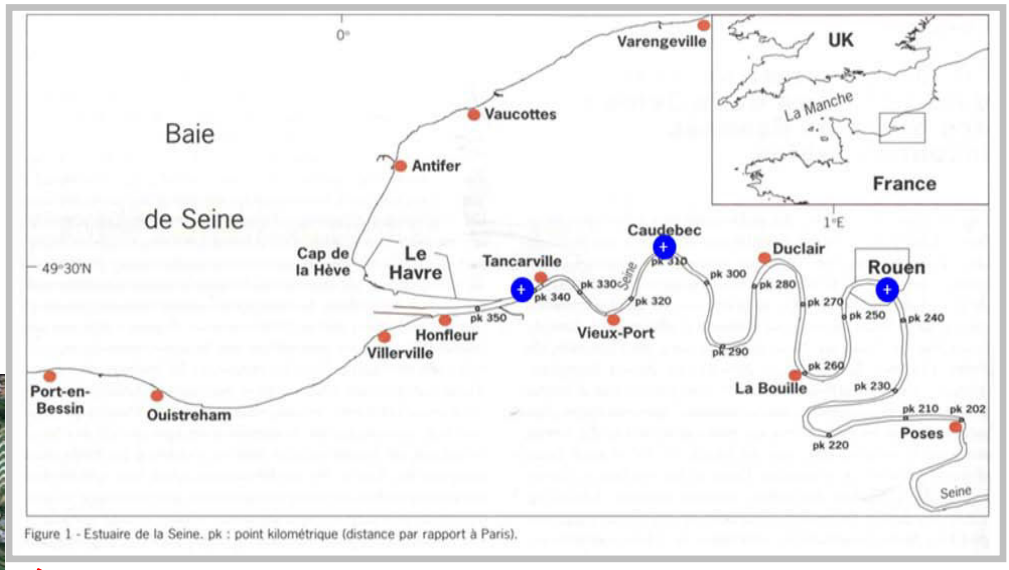
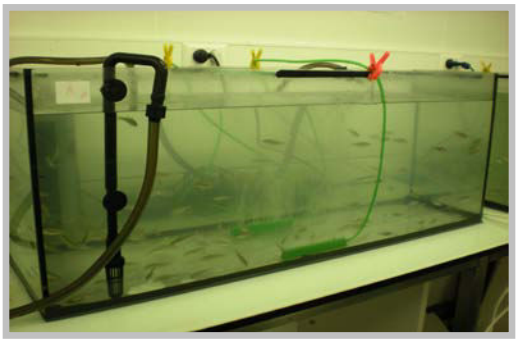



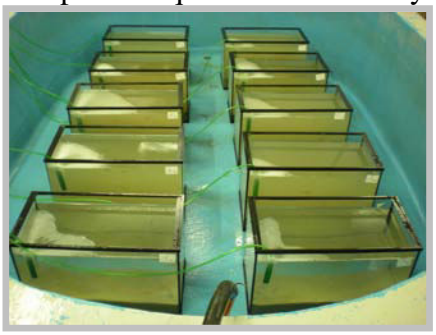
Figure 1 - Estuaire de la Seine. pk : point kilométrique (distance par rapport à Paris).

Juveniles of sea bass from Aquanord hatchery in acclimation aquarium for 2 weeks



Before contamination: Biometric measurement, tagging fish individually and sampling 13 fish for $t_0$

Exposure aquariums for 21 days




After contamination: Biometric measurement, sampling of liver (at $t_7$), muscle and gills (at $t_{21}$) of 100 fish for further analysis

Analysis

- Sediment: granulometry, organic matter contents, metals and its bioavailability, PAHs and PCBs, metals in gills
- Biomarker responses (short term): (EROD, GST, CAT)
- Histology (Tymus and spleen)

Correlation ?

Physiological performance responses (long term): Growth rate in length and weight, RNA-DNA ratio, Fulton's K condition index, mortality and malformations

### II.2.3. Mesocosm experiences on the effects of two toxic algal blooms: *Phaeocystis globosa* and *Pseudo-nitzschia pseudodelicatissima* on the physiological performance of sea bass juveniles (*Dicentrarchus labrax,* L., 1758)

The fish hatchery and farming industry of Aquanord (Gravelines, France), which is specialized in the production of sea bass, is subject each year to recurrent high mortality of both juveniles and adults from March to June, with mortality peaks from the end of May. Interestingly, this period of maximal mortality coincides with the wane of bloom of the prymnesiophyte *Phaeocystis globosa* and the bloom period of the diatom *Pseudo-nitzschia delicatissima* and/or *pseudodelicatissima* along the eastern English Channel seashore.

*P. globosa* has a complex life cycle alternating free cell phase and colonial phase where cells are embedded into a mucilaginous matrix composed of polysaccharides. However, the colonial phase forms the bulk of *Phaeocystis* bloom. *P. globosa* and *P. delicatissima* are classified as harmful algae: *P. globosa* is undesirable mainly because of deleterious effects on tourism and recreational activities that it generates, due to the seafoam events characterizing the end of bloom; *Pseudo-nitzschia* spp. is harmful because of their ability to produce domoic acid, an amnesic shellfish poisoning (ASP) (Zingone and Enevoldsen 2000, Fehling et al., 2005; Sazhin et al., 2007). Seafoam originates from the *Phaeocystis* colony disruption (Lancelot 1995). However, its magnitude and intensity depends on both the *Phaeocystis* bloom magnitude and intensity, but also on wind speed and direction (Lancelot, 1995). Once colonial *Phaeocystis* cells have released from the muccopolysacccharidic matrix, this last then deteriorates and become a Transparent Exopolymer Particle (TEP, Mari et al., 2005). TEP are ubiquitous and abundant sticky substances in the oceans, gel-like particles, ranging from ~1 to several hundred micrometers in size (Passow 2002b).

TEPs play a crucial role in aggregation of particles in the oceans (Alldredge et al., 1993; Mari et al., 2005), and therefore in flocculation processes (Seuront et al., 2006), and seafoam formation (Lancelot, 1995). Therefore, TEPs have been many studied, about their origin, distribution, and fate in the oceans (Passow, 2002a, b). However few studies have explored the effect of these “glue particles” on living organisms, especially fishes. To our knowledge, only zooplankton has been the subject of research to investigate any negative impact on phytoplankton ingestion. TEPs were detrimental for small calanoid copepod feeding (Dutz et al. 2005) whereas they were beneficial to euphausiids (Passow and Aldredge

1999). Net-clogging has been observed during *Phaeocystis* bloom (Savage, 1930; Hurley, 1982; Rogers and Lockwood, 1990; Huang et al., 1999), and fish mortality (Savage, 1930), as well as decline in shell fish growth and reproduction (Pieters et al., 1980; Davidson and Marchant, 1992; Prins et al., 1994; Smaal and Twisk, 1997). These negative impacts of *Phaeocystis* bloom on these living organisms could be due in part to the presence of TEPs.

*Pseudo-nitzschia* blooms are common in marine and estuarine environment and have deep socio-economic impacts on shellfish farming or harvesting and fishermen, but were recognized as being potentially toxic only 20 years ago (Bates et al. 1989; Klein et al., 2010). As it is reported in the literature, pinnate diatoms such as the small needle-shaped *Nitzchia* species are able to reveal abundant populations on *Phaeocystis* colonies and can be capable of producing the toxin as domoic acid on marine organisms such as mussels, filter-feeding shellfish or finfish (Fehling et al., 2005; Sazhin et al., 2007). Members of the genus *Pseudo-nitzschia* have been confirmed as producers of the neurotoxin DA which may enter the food chain from diatoms via filter-feeding shellfish or finfish. The toxin then accumulates to such levels that ingestion of the vectors by humans or other animals may lead to sickness or mortality in sea mammals, seabirds and humans due to ASP. Numerous laboratory-based toxicity studies were performed in order to characterize the neurotoxicity of DA has been observed in several animal species including humans, non-human primates, rodents, rats, fish, marine mammals and birds (Iverson et al., 1990; Tryphonas et al., 1990; Tasker et al., 1991 ; Lefebvre et al., 2001 ; Schaffer et al., 2006). To date, these harmful algae have become a focal point of numerous ecological studies and monitoring efforts in recent years and are subjected of various aspects of DA toxicology, pathology, bioaccumulation, and production in toxic diatom species. (Fehling et al., 2005; Schnetzer et al., 2007; Costa et al., 2010). However, the toxic effects of DA on the growth and condition indices of aquatic organisms, especially of fish juveniles, are scarcely investigated (Dizer et al., 2001; Tiedeken et al., 2005; Lefebvre et al., 2007).

The goal of the present study was to explore the effects either of the mucilage aggregates derived from *P. globosa*, predominantly under the form of Transparent Exopolymer Particles (TEP), as well as freshly foam formed, and *P. pseudodelicatissima* bloom on growth, survival and physiological performances of European sea bass juveniles (*Dicentrarchus labrax,* L., 1758), using various biological and biochemical indices.

### II.2.3.1. Phytoplankton strains and culture conditions

*P. globosa* and *P. pseudodelicatissima* strains were first isolated from the eastern English Channel in autumn 2008 April 2009, respectively, pippeting cells from coastal water samples by Dr. Elsa Breton (LOG, Wimereux)**.** Cultures were maintained in monospecific conditions in f/2 medium (Guillard and Ryther 1962; Guillard, 1975) at 12 ± 0.5°C with a 12:12 h light:dark cycle under a photon flux density of 400 µmol photons $m^{-2}$ $s^{-1}$ (Daylight HQIT-WD 250 W F, OSRAM). For the experiments, a first culture in 10-L and in 12-L Nalgene bottles, for *P. globosa* and *P. pseudodelicatissima*, respectively, were cultivated before to decant it into a 250 L and 100 L plexiglass batches at the hatchery unit of Aquanord, filled up with sea water of *UV*-treated to reduce the risk of pathogenic organism. At this time, culture of *P. globosa* and *P. pseudodelicatissima* were grown at 15 ± 0.5°C (same light conditions) for 2 weeks before starting experiments with sea bass juveniles, in order to allow sufficient TEP production from the degradation of *Phaeocystis* bloom decaying colonies formed on the mucoid polysaccharides fragments to use in the fish tanks (Mari et al. 2005). In addition, freshly accumulated foam and associated TEP were regularly collected at the hatchery pumping site and volume was assessed by gently placing the foam in a large bucket of 46 L. Care was taken not to press the foam so as to preserve its natural aspect and its apparent density. Since, domoic acid production from the diatom species *P. pseudodelicatissima* often starts at the onset of stationary phase of *Pseudo-nitzschia delicatissima* and peaks about one week later in batch cultures (Pan et al., 2001), this species was grown separately for 1 and 2 weeks, in order to test whether *P. pseudodelicatissima* physiological state (exponential versus death phase) affects on juvenile sea bass. For this reason, first batch (250 L) was served as exponential phase where second batch (100 L) was served for death phase that can produce domoic acid.

### II.2.3.2. Phytoplankton experimental procedure

In order to explore the effects of *P. globosa* derived material and *P. pseudodelicatissima* on the growth, survival, and physiological performances of juvenile sea bass, two mesocoms experiments in larval rearing tanks of $1m^3$ were conducted from April to July 2009 at the Aquanord hatchery. For the first experiment, three treatments were applied in the experimental designs for 28 days (from 17 April to 15 May 2009): (1) control (250

individuals), (2) *Phaeocystis* and TEP at starting concentration of 1.16 $10^8$ cells $L^{-1}$ and 7634.3 µg XG eq.$l^{-1}$ (250 individuals in duplicate), (3) freshly 46 L of formed dense seafoam (250 individuals in duplicates). Seafoam was collected with plastic planter at the hatchery pumping station which generates seafoam through turbulence. For the second experiment, 3 treatments were also applied: (1) control (250 individuals), (2) *P. pseudodelicatissima* in exponential phase (*Ps1* and *Ps2*) (250 individuals in duplicate), and (3) *P. pseudodelicatissima* in death phase (*Ps3)* (250 individuals), all cultures at an initial concentration of ~$10^8$ cell $L^{-1}$. These three last treatments were run for 21 days (from 10 to 31 July 2009), except for treatment (3), which started one week later (from 17 to 31 July 2009).

The juvenile sea bass provided by Aquanord were 113 and 103 days old at the start of the experiments for *P. globosa* and *P. Pseudodelicatissima,* respectively. All tanks were supplied with sand-filtered and UV sterilized running seawater, thus permitting to keep mesocosm at *in situ* temperature (15 ±1°C) throughout the experiment. Tanks were illuminated according to the natural photoperiod at a photon density of 400 µmol $m^{-2}$ $s^{-1}$ (Daylight HQIT-WD 250 WF) in a 12:12 h light:dark cycle and gently aerated (compressed air), thus preventing settling of particles and maintaining oxygen saturation above 80%. Fish were fed daily *ad libitum* in the morning with commercial dry pellets (Skretting Ltd., France, Gemma PG 1.0), which contain 56% protein, 18%oil, 10%ash, 0.6% fibre, 1.3% total phosphorus, copper (8 ppm $CuSO_4$), vitamin A (15000 IU/kg), vitamin D3 (1125 IU/kg), and vitamin E (225 IU/kg). In each tank, 25% of the seawater was renewed every two days, therefore, 50 L of *P. globosa* and *P. pseudodelicatissima* cultures added into the each tank to maintain high and stable TEP and *P. pseudodelicatissima* concentrations.

#### II.2.3.3. Sampling

Fifty juvenile sea bass were sampled at the start of the experiment to determine the initial fitness condition of fishes ($t_0$) and to compare those with treatment conditions. Dissolved oxygen concentration (mg/L), temperature (°C), salinity, pH (with Hanna HI 9828 multiprobe) and turbidity (NTU) (with Eutech instruments, TN-100) were measured every 2 days before before seawater renewal and fish feeding. A 40 ml seawater was collected every two days before renewing seawater into the each tank to determine phytoplankton cell abundance for both experiences. TEP and *P. pseudodelicatissima* samples were preserved with formaldehyde (2% final concentration) and with Lugol-gluteraldehyde (1% final

concentration), respectively. All samples were stored at 4°C in the dark until analysis (within one month). In order to monitor growth and physiological performances of juveniles, 30 individuals from each tank were removed on each week and at the end of experiments after being anaesthetised within 3-4 minutes by immersion in 0.32ml/l of 2-phenoxyethanol and stored at -20 °C for further analysis. Finally, fishes remaining in the experimental tanks were observed every two days early in the morning before the first food supply to assess fish mortality. In addition, daily mortality rate was calculated for each treatment during the experiment. In the case of any morphological abnormality or lesion indicating cannibalism, fish were photographed with a digital camera. At the end of *Phaeocystis* experiment, the X-rays were taken of some frozen fish for control and foam conditions. The fish were transferred to plastic bags prior to radiography, performed in a radiology clinic with mammography X-ray equipment. The radiographies of sea bass juveniles were analyzed using TNPC (5.0. NOESIS). The detection of skeletal abnormalities was carried out by visual inspection of the radiographies (Figure 14).

At the laboratory, juveniles were defrosted, measured for SL (standard length) and TL (total length) (near to 0.1 mm) and weighed (wet weight) (near to 0.001 g) to determine growth performance. Beside this, it is calculated the growth rate in length and weight, Fulton's K condition index, RNA-DNA ratio and TAG-ST ratio for sea bass juveniles.

**A** **B**

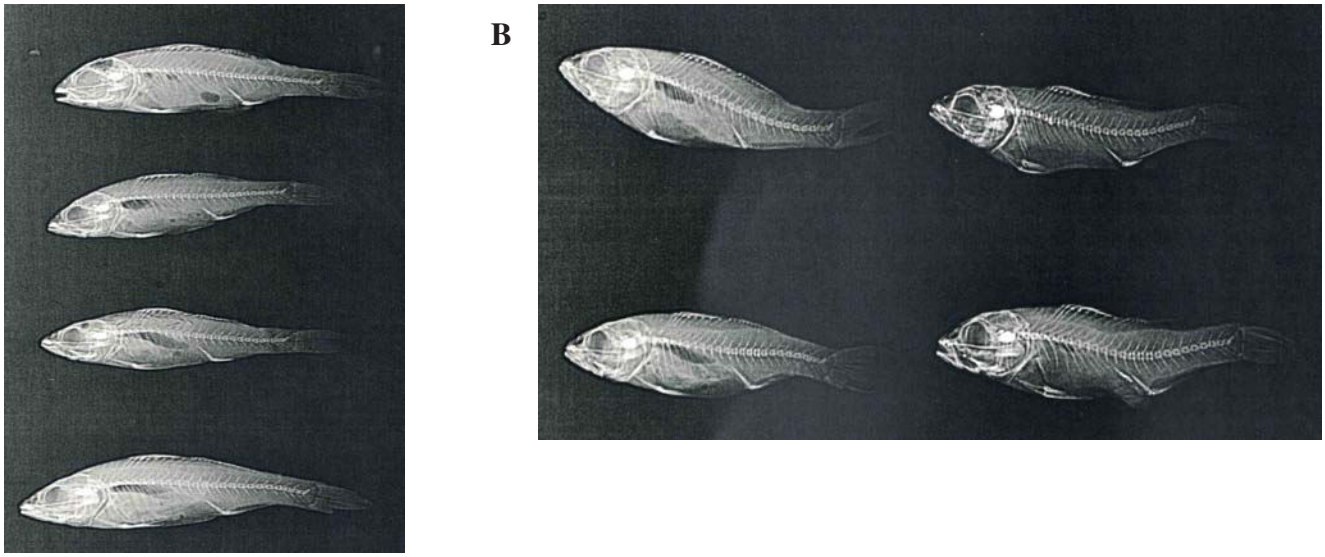
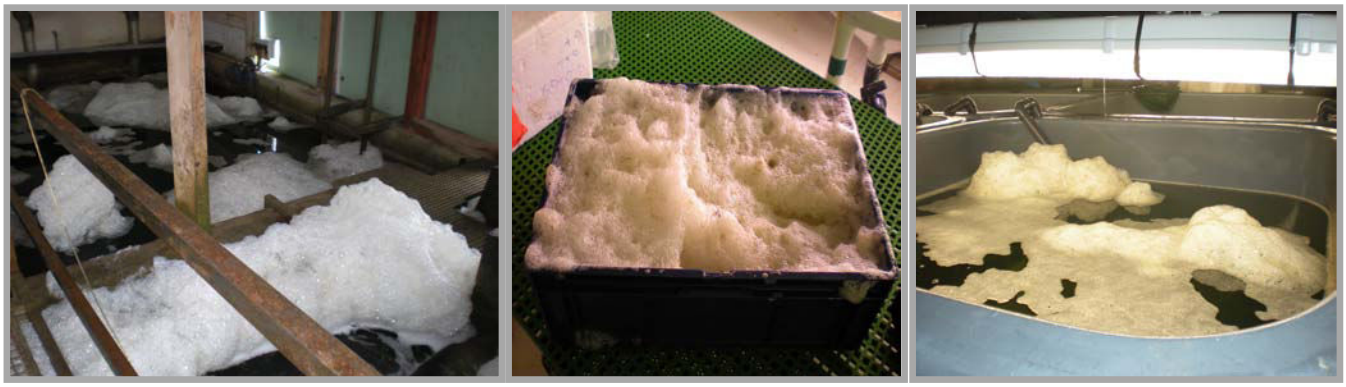


Figure 14. Representative radiographies of sea bass juveniles (A: control fish, B: Foam fish) realised after the 28 days *P. globosa* exposure

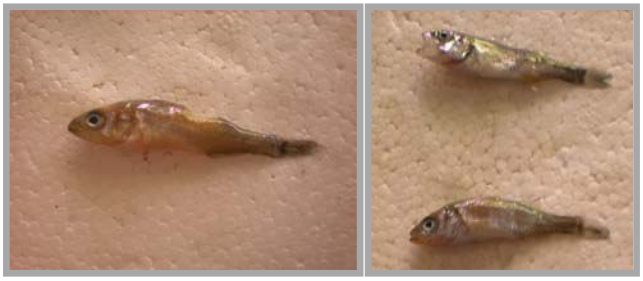
## Second experiment Summary

*Phaeocystis globosa* culture preparation in Aquanord hatchery unit 2 weeks before the experiment and transfer of foam in a case from the hatchery pumping station of Aquanord into foam basins



Acclimatation of sea bass juveniles (113 days) for 2 weeks in $1m^3$ larval basins (n= 250/condition) and sampling of 50 fish for $t_0$ as reference before contamination

Exposure of *Phaeocystis globosa* and foam contamination for 28 days

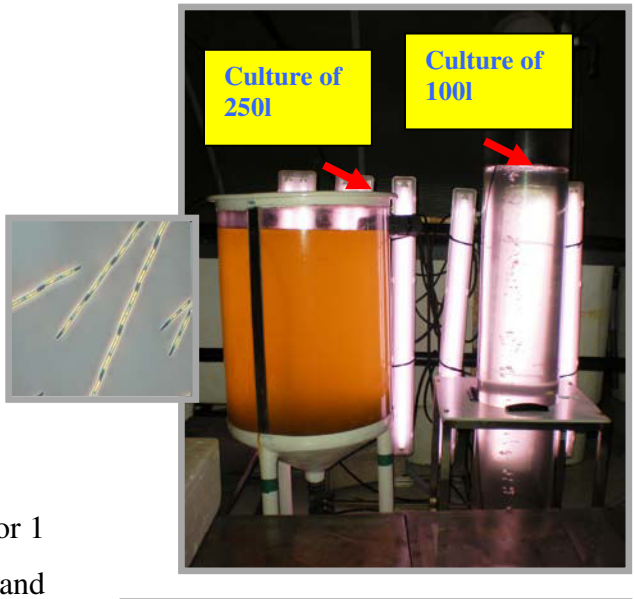



Sampling of 30 fish / condition of $t_8$, $t_{20}$ and the end of experiment

Analysis: growth rate in length and weight, Fulton's K condition index, RNA-DNA ratio (just for $t_0$ and $t_{28}$), observation of mortality and malformations

## Third experiment Summary

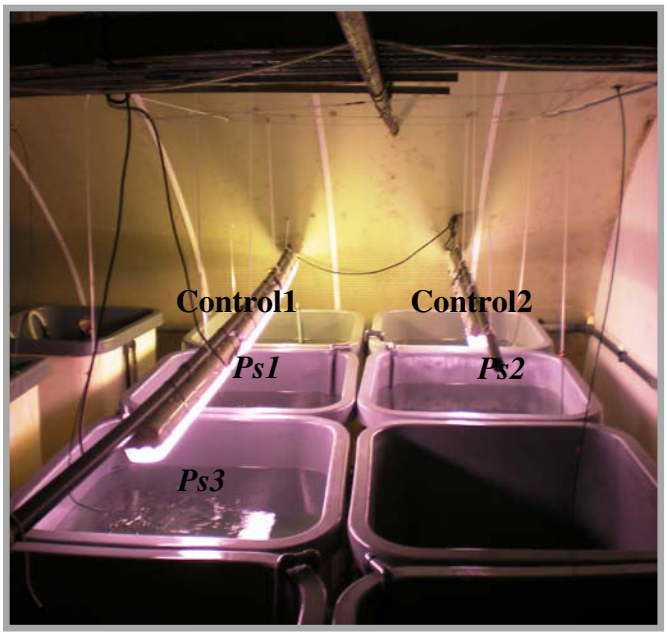
*Pseudo-nitzschia pseudodelicatissima* cultures (exponential (in 250 L) and senescent (in 100 L) phases) in Aquanord hatchery unit 1 week before the experiment




Acclimatation of sea bass juveniles (103 days) for 1 week in $1m^3$ larval basins (n= 250/condition) and sampling of 50 fish for $t_0$ as reference before contamination

Exposure of *Pseudo-nitzschia delicatissima* on exponential and senescent phases for 21 days

Sampling of 30 fish / condition of $t_8$, $t_{15}$ and the end of experiment




Analysis: growth rate in length and weight, relative condition index, RNA-DNA ratio, TAG-ST ratio (just for $t_0$ and $t_{21}$), observation of mortality and malformations

## II.3. Other analysis of *in situ*, microcosm and mesocosm experiences

### II.3.1. Sediment analysis

#### II.3.1.2. Metal analysis

To avoid contamination, all chemical reagents used for the analysis were chosen of Suprapur quality and all materials were intensively cleaned with acid and rinsed with ultra clean water (Milli-Q) before use. Sediments were dried at 40°C to constant weight and were ground to powder using an agate mortar and pestle.

In order to determine total metal concentration (Cd, Cr, Cu, Mn, Ni, Pb, V, Zn, Al, Fe) about 0.3 g of ground sediments were treated in Teflon beakers with 10ml hydrofluoric acid for 48 h at 110 °C. After HF evaporation, sediments were again digested by an aqua regia (6ml hydrochlorydric + 3ml nitric concentrated acids) attack for 24 h at 120 °C. This operation renewed once again. The mixture was partially evaporated and the recovered solutions were diluted at a known volume in MilliQ water.

When measuring bioaccumulation behavior the bioavailability of the substance considered is a crucial parameter for valid results. In a review by Belfroid et al. (1996), the bioavailability was defined as the fraction of the bulk amount of the chemical present in soil/sediment and (interstitial) water that can potentially be taken up during the organism's lifetime into the organism's tissues (excluding the digestive tract). When the concentration in fish is not related to the real bioavailable concentration in the water, this might result in underestimation of the bioconcentration potential (Van der Oost et al., 2003). Some metals associated with the reactive fractions of sediment, considered as bioavailable fraction, were estimated using the method of Huerta-Diaz and Morse (1990). Briefly, the method consists in the extraction of metals associated with exchangeable carbonates partially, -Fe and Mn oxyhydroxides- fractions and to acid volatiles sulfides (AVS) defined previously by Tessier et al. (1979). Thus, about 0.5 g of sediment was leached during 24h with 20 ml of 1 M HCl.

The total and extractable metals were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES;Varian Vista Pro, axial view). For quality assurance, reagents blanks, sample replicates and standard references (HISS-1, MESS-3 and PACS-2, National Research Council Canada) were used to assess the accuracy and precision of the

analysis. In all cases, the recovery efficiency was better than 85 % for the total digestion of these standards.

Total Hg was measured in dry and ground sediment samples (about 50 mg without any pre-treatment) by means of atomic absorption spectroscopy (AAS) using an AMA 254 solid phase Hg-Analyzer (Altec Ltd., Prague, Czech Republic) (Oudanne et al., 2008). Mean recovery for total Hg was better than 95 % for certified estuarine sediments IAEA-405 (IAEA, Vienna, Austria) and MESS 3 (NRC Canada).

#### II.3.1.3. Polycyclic aromatic hydrocarbons and Polychlorinated biphenyls analysis

The persistent organic pollutants, including PAHs (EPA's 16 priority PAHs) and PCBs (7 congeners) were analysed in the laboratory of "Flandres analyses" (France). After the sediments were dried (at 40°C) ground and homogenized an aliquot of 2 g was extracted by microwave with a mix solvent of 20 ml hexane + 20ml acetone. The simultaneous determination of PAHs and PCBs was performed using a Gas chromatography (CP 3800, Varian) by detection of mass spectrometry (1200MS TQ, Varian). The chromatograph was fitted with a 30m * 0.25 mm ID * 0.25µm film thickness of ZB-Multiresidue-1 column from Phenomenex (Torrance, CA, USA). Identification of PAH compounds and PCB congeners was based on the comparison of their GC-retention times and their mass spectrum, with appropriate individual standards.

#### II.3.1.4. Metal analysis: a) in fish and b) in fish gills

a) This analysis was done for the flounder juveniles collected in the four estuaries (Canche, Authie, Somme and Seine) in May-June 2008. Ten flounder juveniles for each estuary were unfrozen at room temperature and their total length and weight were recorded (44.5 ± 6.2 mm; 927.3 ± 387.5 mg). It has been selected the biggest ones to have enough quantity of materiel for metal analysis. Subsequently, whole-body fish samples were lyophilised during 48h. Before acid digestion, an agate employed to grind and to homogenize the dry tissue samples. Approximately 0.5 g of aliquots was digested in Teflon beakers for 12h at room temperature and then 4h at 100°C in hot plate with ~ 4ml nitric acid. After that, the remaining digested solution was diluted with Milli-Q for all elements (Henry et al., 2004).

Concentrations of Cd, Cr, Cu, Mn, Ni, Pb, V, Zn, As and Se were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Varian 820-MS). ICP apparatus were calibrated using standard solutions; blank corrections were applied if necessary and the accuracy of the applied analytical procedure was tested using certified reference materials (DORM-3, fish protein) provided by the National Research Council of Canada.

b) This analysis was done only for the sea bass juveniles contaminated with estuarine sediment in February-Mars 2010. Because of the constraint of gill size, ten fish gills were pooled so two samples of gill were analysed for each condition on $t_0$ and $t_{21}$. The gills were rinsed with Milli-Q water, mixed and lyophilised for analysis of metal concentrations. Approximately 0.08 g of aliquots was digested with ~ 1ml nitric acid (65%, Suprapur Merck) according to Henry et al. (2004). Each metal on gill samples was determined by inductively coupled plasma-mass spectrometry (ICP-MS; VARIAN 820). Standard curves were used to determine Mn and Zn in diluted samples, whereas standard addition technique was applied for resolution of matrix effects to calculate Cd, Cr, Cu, Mn, Ni, Pb, V, Zn, As and Se. International certified standard (DORM–3, NRC Canada) was used to control the accuracy of the analytical procedure.

### II.3.2. Biomarkers

In our research, the liver of sea bass juveniles were sampled in experimental study to detect the effects of estuarine sediment contamination on the physiological performances from relationship between short term (biomarkers) and long terms (biological indices) responses.

Biomarkers are defined as observable or measurable modifications at the molecular, biochemical, cellular, physiological or behavioural levels revealing the exposure of an organism to xenobiotics (Lagadic et al., 1997). They have been proposed as sensitive tools for detecting environmental exposure and adverse effects of toxic anthropogenic chemicals on aquatic organisms (McCarthy and Munkittrick, 1996; Sanchez et al., 2007). They can be used to examine pollutants stress in organisms and to assess the health status of organisms and to obtain early-warning signals of environmental risks. Since many of the biomarkers are short-term indicators of long-term adverse effects, these data may permit intervention before irreversible detrimental effects become inevitable (Van der Oost et al., 2003).

Generally, the most sensitive effect biomarkers are alterations in levels and activities of biotransformation enzymes. In fish, the activity of these enzymes may be induced or inhibited upon exposure to xenobiotics. In addition, many environmental contaminants (or their metabolites) have been shown to exert toxic effects related to oxidative stress. With respect to neuromuscular functions, recent studies indicated that the 'old' biomarker acetylcholinesterase (AChE), which is sensitive to organophosphate (OP) and carbamate pesticides, may be responding to low levels of contaminants in the environment (Van der Oost et al., 2003). Beside this, in fish, the class of cyt P450 isozymes which is responsible for the biotransformation of a myriad of xenobiotic compounds (PAHs, PCBs, dioxins, etc.) is the CYP1, 2 and 3 subfamilies (Hasselberg et al., 2004). Moreover, among the biochemical biomarkers described in the literature, phase I and phase II biotransformation parameters such as EROD (ethoxyresorufin-Odeethylase) and GST (glutathione-S-transferase) activities are currently used in environmental risk assessment (Sanchez et al., 2008). The activity of ethoxyresorufin O-deethylase (EROD), however, appeared to be the most sensitive catalytic probe for determining the inductive response of the cyt P450 system in fish (Goksøyr and Förlin, 1992). Recently, an extensive review was published, compiling and evaluating existing scientific information on the use, limitations, and procedural considerations for EROD activity in fish as a special biomarker of chemical exposure for polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs) (Whyte et al., 2000). In addition, the susceptibility of different fish species to chemical carcinogenesis may be modulated by the activity of GST (Varanasi et al., 1987). There is particular interest in antioxidant enzymes that oppose ROS-induced oxidative damage. Thus, these enzymes are also commonly used to understand the associated toxic-mechanisms of xenobiotics (Sanchez et al., 2005; Oliveira et al., 2008). Catalase, which protects tissues against damage by hydrogen peroxide, was one of the first enzymes proposed to be an effective marker of oxidative stress (Livingstone et al., 1993).

In this context, we studied some biomarkers that were related to both antioxidant (oxidative stress) (Catalase: CAT) and detoxification enzymes (7-ethoxyresorufin-O-deethylase: EROD and Glutathione S-transferase: GST) in the liver.

II.3.2.1. Standard and samples preparations

Livers were removed, weighed and frozen in liquid nitrogen prior to homogenization and biochemical analysis. The homogenization was in an ice-cold phosphate buffer (0.1 M, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were ground with a ball mill at 6000 rpm during 2*8 secondes (Precellys, Bertin Technologies, France), centrifuged at 10.000 g at 4 °C, for 15 min (EBA 12R, Hettich centrifuge, Tuttlingen, Germany) and the supernatants that represented post-mitochondrial fractions were used for biochemical assays. Total protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard. SOD was assessed according to the methods of Paoletti et al. (1986); using purified bovine enzymes (Sigma) as standards. For GST activity determination, chlorodinitrobenzene was used as substrate (Habig et al., 1974) and purified GST from equine liver (Sigma) as a standard. 7-Ethoxyresorufin-O-deethylase activity was determined by a fluorimetric method in black microplates (Flammarion et al., 1998) using a microplate spectrofluorimeter reader (Victor2 Wallac, Perkin-Elmer). These assays were adapted in microplates and optimised for European sea bass to fit the linearity of each assay. The total protein concentrations used for EROD activity were chosen 0.5 to 10 g/l for this species. All measures were carried out at room temperature in microtiter plates, using a microplate reader (Packard BioScience Multiprobe® II HT EX Liquid Handling system with Gripper integration platform) (Figure 15). Samples were quantified by fluorimetric measurement with 515 nm wavelength excitation and 553 nm wavelength emissions. All these measurements were realized in the laboratory of INERIS (Unité d'écotoxicologie in vitro et in vivo, Institut National de l'Environnement Industriel et des Risques, Verneuil en Halatte, France).

**Grinding of liver samples** ⇨ **Centrifugation** ⇨ **Samples measurement**

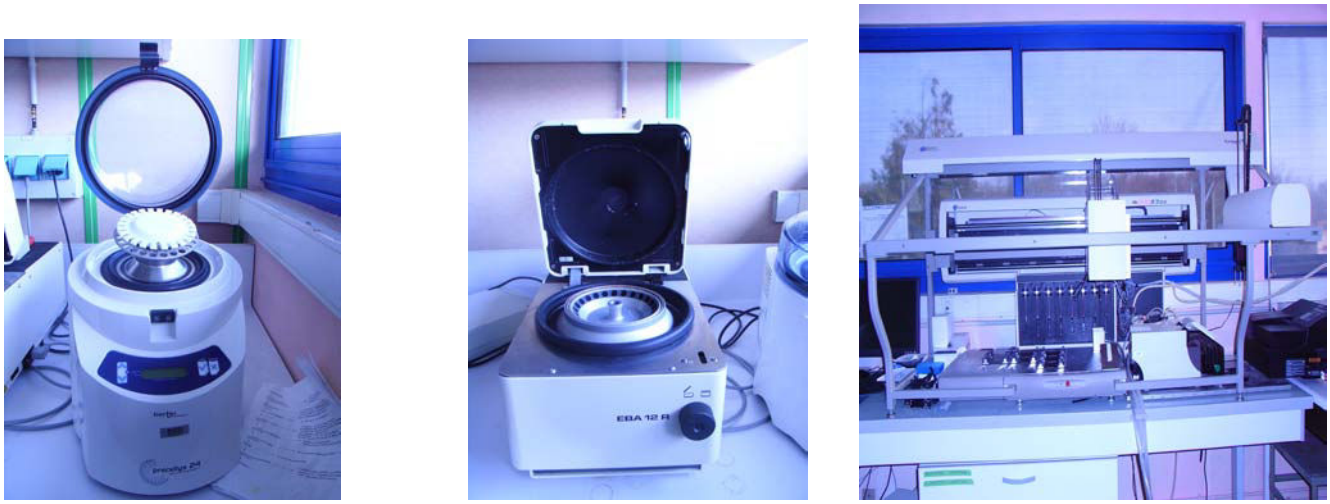


Figure 15. Grinding protocol of fish samples liver

## II.3.2.2. Biotransformation (detoxification) enzymes

- Ethoxyresorufin-O-deethylase activity (EROD) was determined by a fluorimetric method into microplate following the hydroxylation of 7-ethoxyresorufin by the method of Flammarion et al. (1998). 10µL of diluted sample (concentration must be between 0.5 and 10 µg/L) is mixed with 200 µL of reaction mixture. The reaction mixture consisted of phosphate buffer (0.1 M, pH 6.5), 7-ethoxyresorufin (8 µM) and NADPH (0.5 mM). The change in fluorescence was recorded during 30 minutes (excitation wavelength 530 nm, emission wavelength 585 nm) and enzyme activity calculated as $pmol.min^{-1}.mg^{-1}$ protein using resorufin standard.

- Glutathione S-transferase activity (GST; EC 2.5.1.18) was determined following the conjugation of reduced glutathione with chloro dinitro benzene (CDNB) by the method of Habig et al. (1974). 10 µL of standardized sample with 0.6 g/L are mixed with 133 µL of reaction mixture. The reaction mixture consisted of phosphate buffer (0.1 M, pH 6.5), reduced glutathione (1 mM) and CDNB (1 mM). The change in absorbance was recorded during 6 minutes at 340 nm and enzyme activity calculated as $\mu mol.min^{-1}.mg^{-1}$ protein using GST standard.

II.3.2.3. Antioxidant enzymes (oxidative stress biomarkers)

- Catalase activity (CAT; EC 1.11.1.6) was determined by the method of Babo and Vasseur (1992). Briefly, the assay mixture consisted of phosphate buffer (100 mM pH 6.5) and $H_2O_2$ (28 mM). Change in absorbance was recorded at 240 nm at 25°C for 1-2 minutes. CAT activity was calculated in terms of U/g and/or μmol.min$^{-1}$.mg$^{-1}$ protein using bovine erythrocyte Catalase as standard.

### II.3.3. Fish mortality and physiological fish performance indicators

Measures of growth and condition indices of young fishes have been used to assess the effects of environmental conditions on individuals (e.g. Suthers et al. 1992). These indices reflect the habitat in which the fish live by using short-and mid-term indices (e.g. RNA-DNA ratio; recent growth index). In this context, following analysis were applied for flounder and sea bass juveniles in order to determine their physiological performance against the different environmental stressors.

II.3.3.1. Daily mortality

Mortality of European sea bass juveniles were recorded during the algal contamination mesocosm experiments in 2009. Dead fishes were counted daily and instantaneous daily mortality coefficients (M, day$^{-1}$) were estimated for each condition and tank applying the exponential model of decline:

$$Nt = N_0 \exp^{-M\,(t-t0)}$$

where $N_t$ is the number of fishes at time $t$. $N_0$ is the number of fishes at the beginning of the experiment ($t_0$) , $(t-t_0)$ is the experiment duration (days), and M is the instantaneous daily mortality coefficient (Ricker, 1958).

II.3.3.2. Biological analysis

At the laboratory, fish juveniles were defrosted, measured for SL (standard length) and TL (total length) to the nearest 0.1 mm and weighed (wet weight) to the nearest 0.001 g to determine their growth performance. Examination of external pathological abnormalities such

as disease, fin erosion was observed in flounder and sea bass juveniles. Nevertheless, it was recorded also the position of the eyes in flounder juveniles (right or left).

### II.3.3.3. Specific growth rates in length and weight

Specific growth rates in weight (mg $d^{-1}$) were estimated as $GW = 100 * (lnW2 - ln\ W_1) / (t_2-t_1)$, where $W_1$ and $W_2$ are mean fish total body weight at times $t_1$ (beginning of the experiment) and $t_2$ (time of collection). Similarly, the specific growth rates in length ($mm.d^{-1}$) was estimated as $GL = 100 * (lnL2 - ln\ L_1) / (t_2-t_1)$, where $L_1$ and $L_2$ are mean fish total length at times $t_1$ and $t_2$ (Ricker, 1979).

### II.3.3.4. Morphological condition index

Fulton's condition factor, K, (Fulton, 1911) has been used as an indicator of the general well being or fitness of fish for this study. This latter morphometric index assumes that heavier fish for a given length are in better condition (Sutton et al., 2000) and was calculated with the following formula: $K$ (mg. $mm^{-3}$) $= (W / L^3) * 100$, where $W$ is the gutted body weight (mg) and $L$ is the total length (mm). TL was measured from the tip of the snout to the end of the tail fin for each fish. In addition, relative condition index was also determined for some experiments on juveniles of European sea bass according to the formula $K\ (\%) = ((K_2-K_1) / K_1) * 100$, where $K_1$ is mean initial condition index (beginning of the experiment) and $K_2$ is mean final condition index (time of collection).

This index is obtained from some morphological measurements (size and weight of fish) and is thus fast and easy to calculate and inexpensive. It is considered as representative of the nutritional state and reserve of energy of individual utilized during periods of starvation, reproduction, and maturation (Lambert and Dutil, 1997; Robinson et al., 2008; Sutton et al., 2000).

Fulton's K index is based on the allometric relation which binds the size and the weight (Suthers, 1998), written in the form: $W = aL^b$ where $W$ is the weight (mg), $L$ the total length (mm), $a$ and $b$ are constants and supposes as an isometric growth (b =3) (Bolger & Conolly, 1989).

We checked this relation in order to validate its use. In addition, this index can involve a skew for the comparison of individuals belonging to different ranges of size. That is avoided

in our case because the totality of the analyzed individuals for this study, that is, flounder, sea bass or three-spined stickleback is of identical class of size.

### II.3.3.5. Growth index

Otolith growth is a complex phenomenon integrating various factors that can be considered either as endogenous or exogenous, although they are always regulated by the physiology of the fish. Both types of factors may operate upon the anabolism and catabolism of the fish. Otolith formation involves rhythmic variations in the deposition and size of organic matrix fibres and carbonate crystals, resulting in the formation of macroscopic translucent and opaque rings and microscopic zonations (growth increments). For these structures to be of use in age estimation, they must be regulated by an endogenous rhythm linked to a periodic environmental cycle or synchronized to periodic events.

The patterns of daily growth of otoliths during early development, in relation to environmental factors, have been studied by time-series analysis of increment width data. These studies deal in several ways the changes in otolith growth rate due to fish size, which confounds the underlying otolith growth (Morales-Nin, 2000). Many studies have demonstrated that the relationship between otolith size and somatic size depends upon growth rate (Reznick et al., 1989; Secor and Dean, 1989, 1992; Casselman, 1990; Mugiya and Tanaka, 1992). This dependency biases traditional approaches to back-calculation and in fish of the same size results in larger otoliths in slow-growing individuals. This phenomenon uncouples somatic and otolith growth rates and obscures the use of daily increment widths as a measure of somatic growth rate (Mosegaard et al., 1988; Campana, S.E., 1990).

In this study, otolith microstructure analysis was used to estimate daily growth increments on fish juveniles. Otoliths were analysed in order to estimate the recent growth index and to explore the otolith–fish size relationship as a tool for evaluating growth differences between estuaries and experimental conditions (as suggested by Secor and Dean, 1989). In fact, results from several studies (Secor and Dean, 1989, Wright et al., 1990; Hare and Cowen, 1995) suggest that slower-growing fish may in fact have larger otoliths than faster-growing fish of similar sizes or ages. Otoliths of flounder (*Platichthys flesus*) and sea bass (*Dicentrarchus labrax*) juveniles were analysed to measure the growth index. Both sagittal otoliths of each fish were removed, cleaned in distilled water, mounted with the convex side (sulcus) up on standard microscope slides, embedded in glue (cyanoacrylate), stored dry and coded (Figure

16). The otolith surface was polished with grinding paper of decreasing grit sizes (5 to 0.1 µm) until increments at the outer edge were visible. The polishing process was controlled frequently under the microscope. The right and left sagitta separated and photographed to measure the diameter and perimeter of each otolith. Since there was no significant difference in the parameters measured between the two sagittae (ANOVA, $p>0.05$), only the right sagitta was used in the analyses. It was also measured some other parameters on the right and left otoliths such as width and surface.

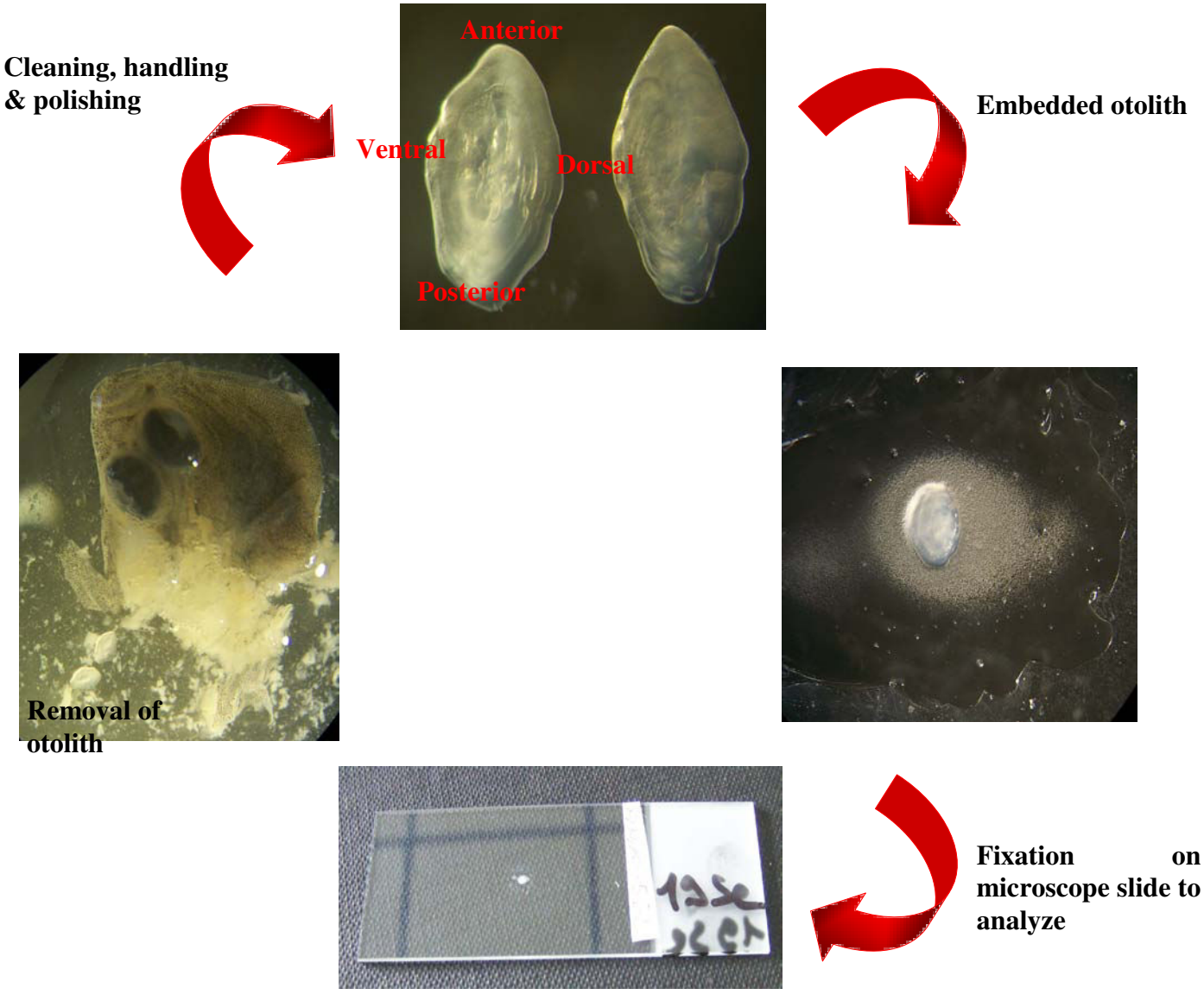



Figure 16. Process of otolith preparation for microstructure analysis

The recent growth index (RG; µm) was determined by measuring the width of the peripheral daily increments of the otoliths. Because there was a significant relationship between sagittal diameter and fish length (Figure 17). We used daily otolith increments from the last 5 days before capture as an indicator of growth (mean distance between the margins of the otolith back to the 5th ring) (Figure 18). We made all increment width measurements as often as possible along the antero-posterior axis (Figure 19) (Amara et al., 2009). Otoliths were analysed under transmitted light at 40 magnifications, using a video system fitted to a compound microscope. All measurement was done along the same axis using an Image Analysis System (TNPC 5.0. © Noesis)

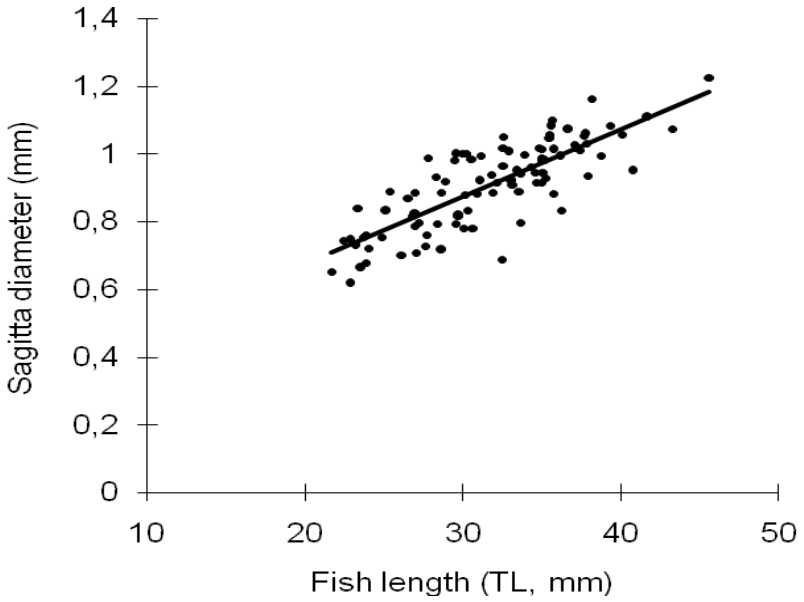



Figure 17. The relationship between fish TL (mm) and the sagittal otolith diameter (µm) in 0-group flounder. Regression model: diameter = 0.0196 + 0.286 (TL); n = 94. $R^2 = 0.63$, $p < 0.001$

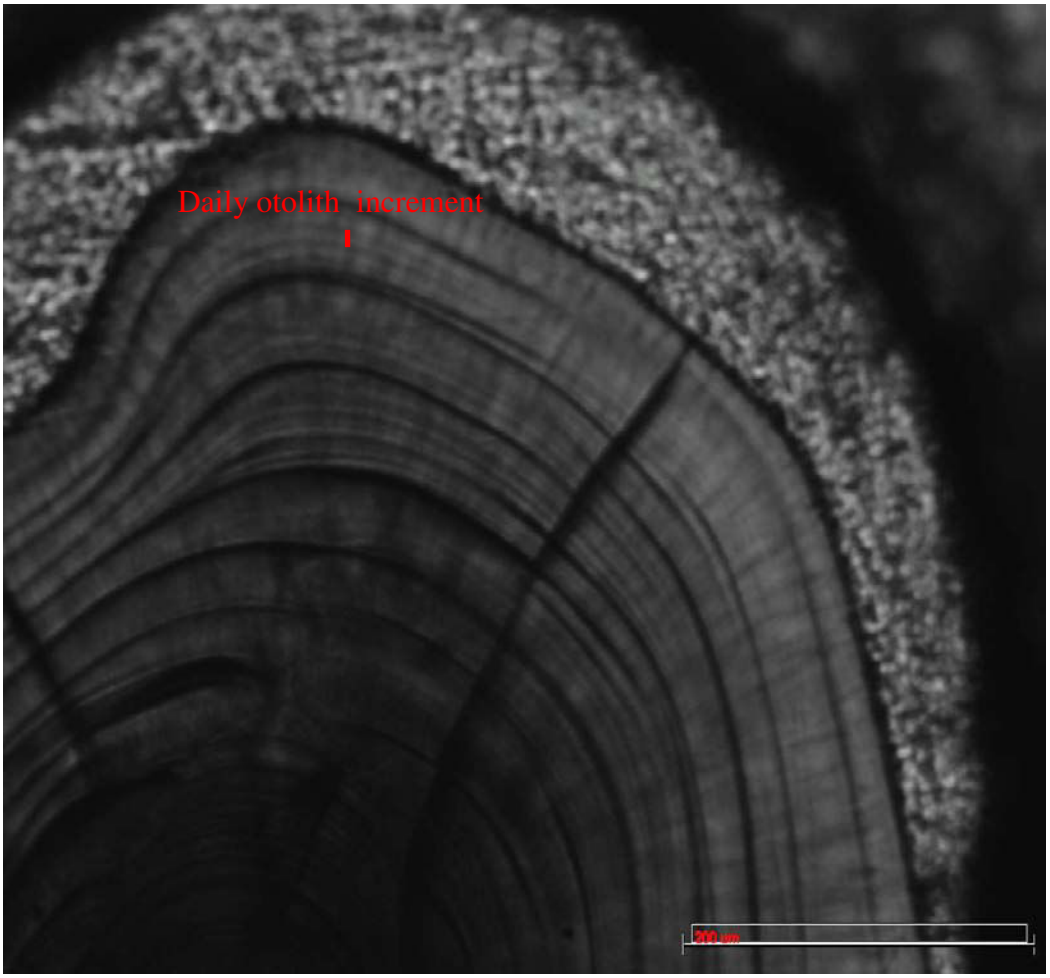



Figure 18. Daily otolith increments in a sagittal section of flounder

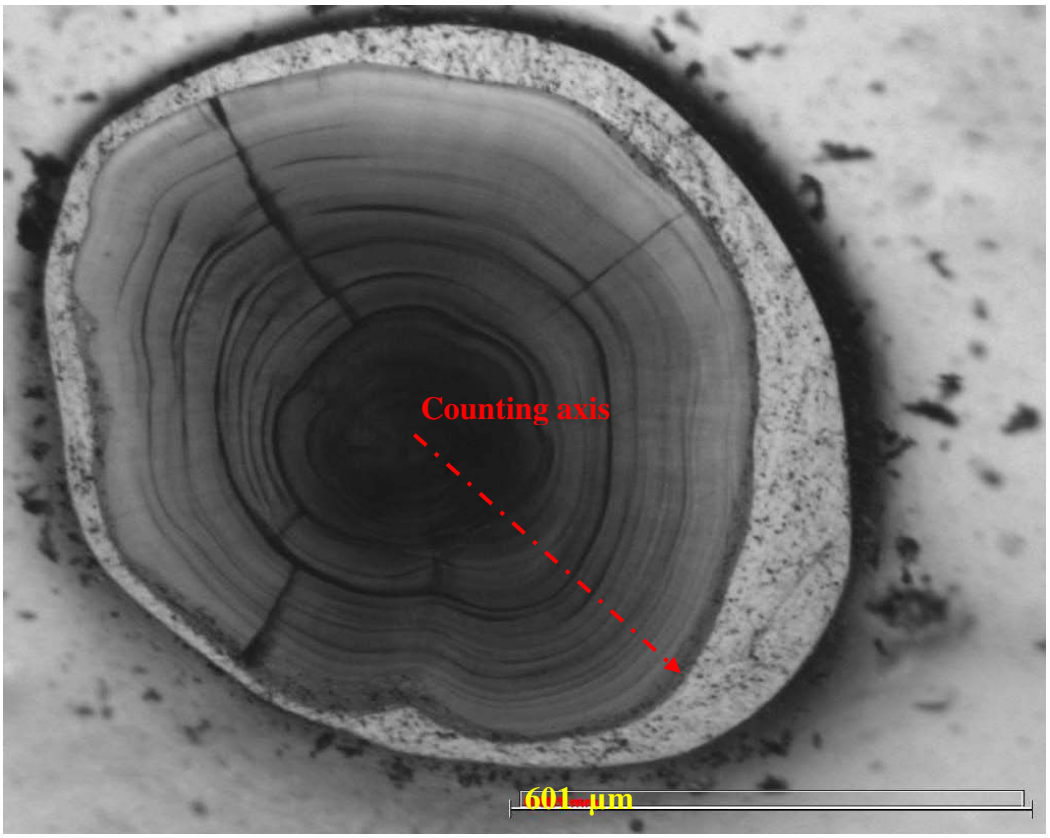



Figure 19. Sagitta of juvenile flounder after polishing observed under optic microscope and orientation of counting axis

### II.3.3.6. Nutritional indices

#### II.3.3.6.1. TAG/ST ratio

Lipides indices have been used to evaluate environmental stressor effects in fishes (Alquezar et al., 2006). In this study, it has been used this lipid storage index based on the ratio of the quantity of triacylglycerols (TAG; reserve lipids) to the quantity of sterols (ST; structural lipids) in the fish. This ratio (TAG / ST) was selected as an index of nutritional status. The TAG content is dependent on the nutritional state of the fish as they are a principal reserve of energy in teleosts and the first components to be mobilised during periods of stress, while sterol contents are essential for the formation of cell membranes and in controlling membrane fluidity and remain essentially unchanged during starvation (Fraser, 1989; Galois et al. 1990; Häkanson et al., 1994; Hilton et al., 2008). Lipid extraction was conducted using the method of Bligh & Dyer (1959) slightly modified by as described by Amara et al. (2007).

The amount of total lipids in each individual was measured on a sample of lyophilised muscle (0.07 g, dry weight). Lipids were extracted from the sonicate for 20 min. ($T < 40°C$) with a solvents mixture of distilled water:methanol:chloroform (1:2:1, v/v/v) (HPLC grade, Fisher Scientific, Pittsburgh, PA, USA). Addition of distilled water:chloroform mixture (1:1, v/v) formed an aqueous-organic 2-layer system. Lipids were transferred into the lower chloroform phase and the transfer improved by centrifugation (3000 rpm, 5 min). The aqueous phase was re-extracted one more time. The separated chloroform layers were combined, rotary evaporated and then dried under nitrogen. After evaporation under a stream nitrogen, the crude of total lipid fraction was weighed ($mg.g^{-1}$, dry weight), redissolved in chloroform:methanol (2:1, v/v) and stored in glass vials (Chromacol Ltd.) at -20°C until used for analysis. TAGs and sterols were separated from other lipids by performing thin layer chromatography (TLC). The lipid extracts of each sample and three blank for detection of any contamination were injected 2 times (10µl) using a capillary (Camag, Switzerland) on Silica gel-60 plates on glass (10 cm \ 10 cm, Merck, Darmstadt, Germany). Each plate is introduced into a tank containing a solvent mixture of hexane:diethyl ether:glacial acetic acid (16:4:0.2, v/v/v) for 15 min. using two chromatography papers (MN 260, Macherey-Nagel, GmbH & Co. KG) to support the migration. After this step, the chromatogram plate is immersed in a fixing solution (phosphoric acid: acetic acid (33%): sulphuric acid: copper sulphate (0.5%) (20:20:2:360, by vol.) for 1 minute. The chromatogram is then dried with the hot air and the

fixed lipidic classes are revealed on hotplate at120°C for 30 minutes. The chromatograms were then scanned by an image scanner (GT-15000, Epson, Tokyo, Japan) with a gray scale mode in 800 dpi and recorded as JPEG format. The software used for scanning was Adobe Photoshop (Adobe Systems Inc., San Jose, USA). The relative contribution of each lipidic class by integrating the surface of the chromatogram concerned were determined on the basis of band intensity by an image analysis program (image J 1.43u) of the National Institute of Health. With this method it is possible to determine six lipidic classes (phospholipids, sterols, free fatty acids, triglycerides, waxes, sterols esters) of which the quantity is expressed in µg/g dry weight (Figure 20). Ratio TAG/ST can then be calculated for each sample. This index is measured only individually for sea bass juveniles exposed to *Pseudo-nitzschia delicatissima* in 2009 experimentally.

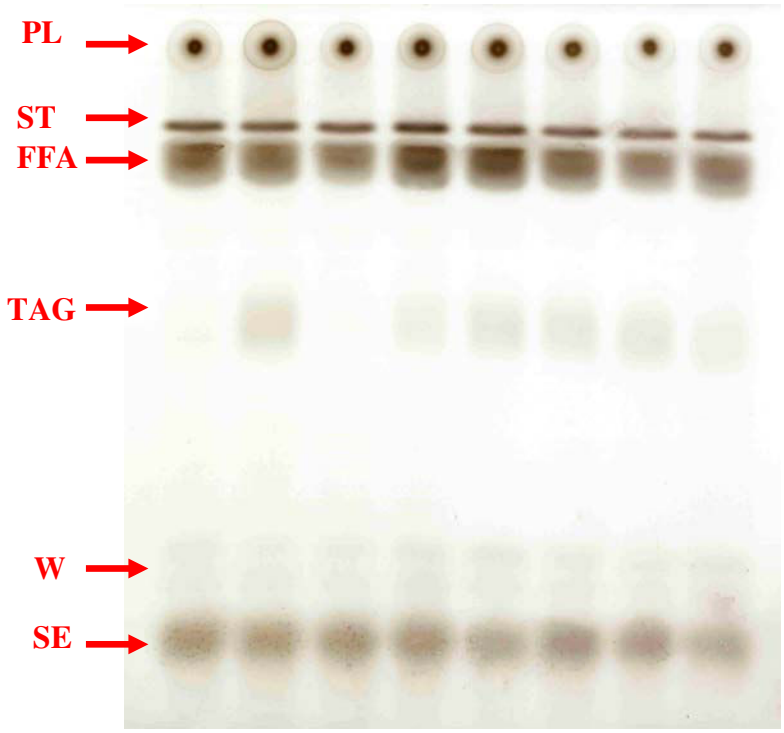



Figure 20. Six lipid class on TLC plate (PL: Phospholipids, ST: Sterols, FFA: Free fatty acids, TAG: Triacylgylcerol, W: Waxes and SE: Sterol esters

II.3.3.6.2. RNA/DNA ratio

Nucleic acid quantification and subsequent RNA-DNA ratios has been used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Richard et al., 1991; Gwack and Tanaka, 2001). This biochemical index reflects variations in growth related protein synthesis, since the quantity of ribonucleic acid (RNA) varies with the rate of protein synthesis, while the amount of desoxyribonucleic acid (DNA) per cell is species-constant in somatic tissue (Buckley and Bullow, 1987). Methodology used to determine RNA and DNA concentrations in individual fish follows the protocol of Clemmesen (1988), based on the method of extraction and quantification of Munro & Fleck (1966). Head and fins were discarded before analyzing fish and guts were excised to ensure that gut contents did not contribute to RNA-DNA ratio. Dissecting tools were rinsed with deionised water between sample dissections to avoid contamination. It was sampled individually a fragment of muscle (±50 mg) for each fish to use for the extraction of this analyse. This sample was homogenized in 300 µl icecold (around 0°C) Tris-buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, 0.2 $mg.ml^{-1}$ proteinase K, pH = 7.5) using an Ultra-Turrax. 30 µl 20% SDS was added and sample was mixed and centrifuged. Then, 300 µl of phenol and 300 µl of chloroform-isoamylalcohol (24:1) were added to supernatant before mixing and centrifugation. This was carried out twice. The supernatant was then discarded, washed twice with chloroform-isoamylcohol and mixed and centrifuged. The resulting supernatant, containing nucleic acids, was mixed with 200 µl Tris-buffer. 5 µl of ethidium bromide (EB, specific fluorophor of nucleic acids) was added to 45 µl of sample to determine both RNA and DNA fluorescence. The fluorescence was determined by exciting at 365 nm and reading the emission at 590 nm using a spectrofluorometer. An aliquot of the sample was then treated with 0.3 µl ribonuclease A (10 $mg.ml^{-1}$), incubated at 37°C for one hour and the fluorescence measured, which is assumed to be due to DNA. The fluorescence due to RNA was then calculated as the difference between total nucleic acids fluorescence and fluorescence after ribonuclease treatment. Standard calibration curves (fluorescence = f [nucleic acid concentration]) for RNA and DNA were beforehand determined with a series of dilutions of pure Salmon sperm DNA (Sigma-Aldrich Chemicals, France) and yeast type III RNA (Sigma-Aldrich Chemicals, France). Fluorescence in RNA and DNA are then converted into concentration expressed as nanograms per µl using calibration curves. The DNA and RNA calibration curves are presented in Figure 21. RNA-

DNA ratio was calculated by dividing RNA to DNA value. Both RNA and DNA concentrations indicated significant correlations with Ethidium Bromide (EB) fluorescence ($p < 0.001$). The curves were fitted by linear regression as follows:

DNA : $y = 24.920x$, $R^2 = 0.97$ ; RNA : $y = 20.599x$, $R^2 = 0.99$

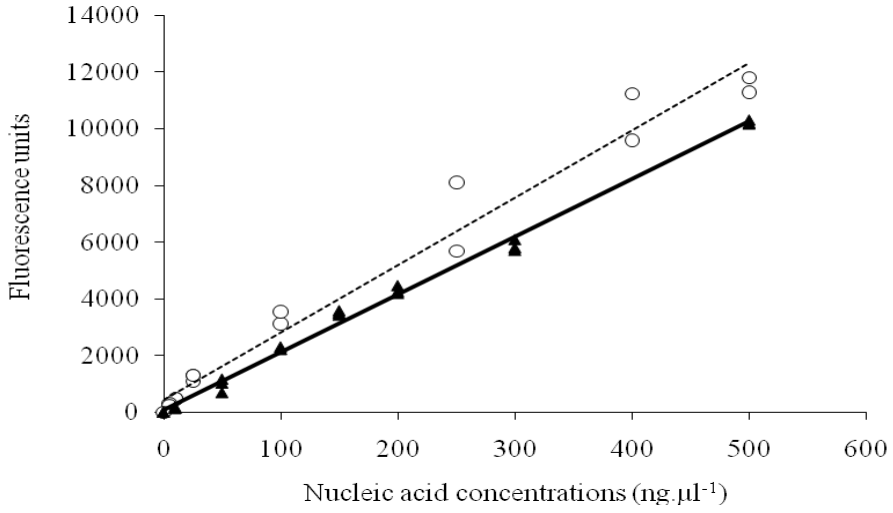



Figure 21. [DNA] ○ and [RNA] ▲ standard curves

### II.3.4. Histology

In cooperation with the LEMA (Laboratoire d'Ecotoxicologie-Milieux Aquatiques) Of the University of Le Havre the thymus and spleen of juvenile sea bass were examined for histological analyses in order to detect the impact of sediment released mixture of pollutants on the immune organ structure and size.

Due to the tiny thymus size, the head section containing the thymic tissue was sampled entirely, while the spleen was dissected apart. The tisssus were either directly fixed in buffered 4% Formaldehyde – Solution (spleen) or stored in Ethanol (100%) for 7 days (thymus) before further dissection and fixation in buffered 4% Formaldehyde – Solution. The head section including the thymus was decalcified in 22% formic acid solution for 5 days (Figure 22). Afterwards, the tissues were dehydrated automatically in a tissue processor (Peloris, Medite, Nunningen, Switzerland) and embedded in paraffin. 5µm thick sections were

cut with a microtome (Leica) and transferred to poly-L-lysine (Sigma, France) coated glass slides. Machine-supported (COT20, Medite) HES (Haematoxylin-Eosin-Safran) stained cuts were observed with Leica QWin Version 2.5.

The number of melanomacrophage centers per area in spleen (10 random cuts per 5 fish of each treatment) was investigated. In thymus, the volumes of the whole organ, cortex and medulla ($mm^3$) and the ratio cortex /medulla were determined. Both thymi per fish (n=5) were examined and a section every $100^{th}$ µm was studied (Casteleyn et al., 2007).

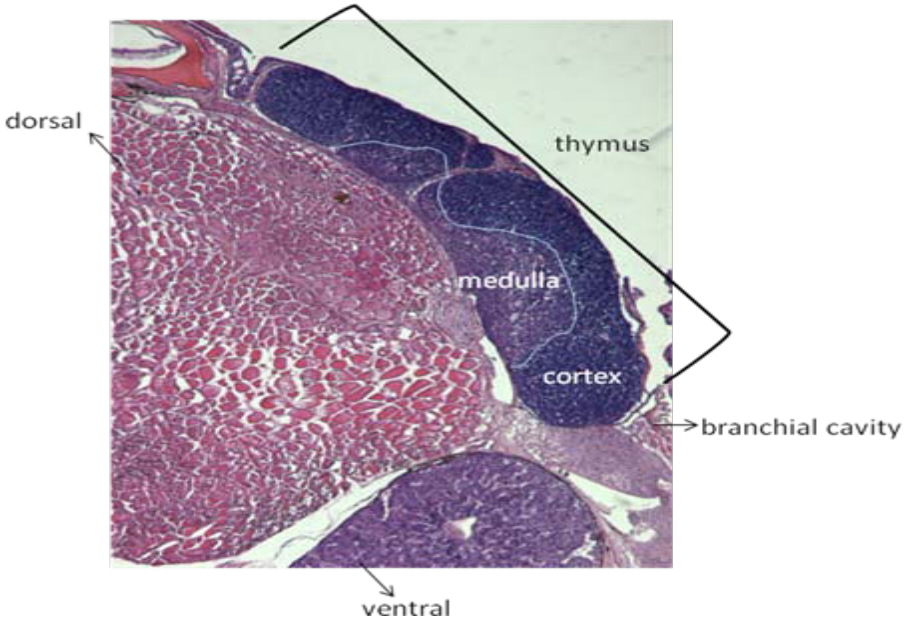



Figure 22. Transversal section through the anterior section of *D. labrax* head (50x); the regionalization of the thymus into cortex and medulla

### II.3.5. Analysis of two algal blooms: *Phaeocystis globosa* and *Pseudo-nitzschia pseudodelicatissima*

In order to determine the concentrations of the mucilage aggregates derived from *P. globosa*, predominantly under the form of Transparent Exopolymer Particles (TEP), as well as freshly foam formed, and the total abundances of *P. pseudodelicatissima* during two mesocosm experiments, it was used following methods.

II.3.5.1. Colorimetric method analysis for transparent exopolymer particles (TEP)

TEP concentrations were determined according to the colorimetric method of Thornton et al. (2007). Briefly, 12 ml of seawater preserved sample were first dialyzed for 24 h with 1000 Da Molecular Weight Cut Off (MWCO) to remove salts which may interfere with the binding properties of Alcian blue. The following day, dissolved Acid Polysaccharides (APS) were separated from TEP by filtering 7 ml of the dialyzed filtrate at low vacuum (< 0.1 bar) onto 0.2 µm polycarbonate Nuclepore filters. APS were then stained with 1 mL of Alcian Blue stock solution (0.02% Alcian blue in 0.06% acetic acid; pH adjusted to 2.5; Passow and Alldredge 1995a, b). Stained APS precipitates were retained on a syringe filter containing a surfactant free cellulose acetate (SFCA) membrane with a pore size of 0.2 µm (Nalgene). Finally, the amount of dye remaining in the filtrate was determined spectrophotometrically at 610 nm and was inversely proportional to APS concentrations. The remaining 5 ml dialyzed filtrate was directly stained with Alcian Blue to bind both dissolved APS and TEP and then filtered on SFCA syringe filter. TEP concentrations were deduced from the difference of absorbance between the pools [TEP+APS] and [APS] and expressed as Gum xanthan equivalent per liter (GX eq $L^{-1}$).

II.3.5.2. Sampling and determination of *Pseudo-nitzschia pseudodelicatissima* total abundances

During the experience of contamination from *Pseudo-nitzschia pseudodelicatissima*, some 40ml seawater was collected every two days before renewing seawater into the each tank to determine phytoplankton cell abundance. In addition, we used the database of Aquanord for the concentration of *P. pseudodelicatissima* in their fish farm basins and their mortality (% $d^{-1}$) during the same period of our experiment to compare the values. Phytoplankton samples were preserved with Lugol-gluteraldehyde (1% final concentration) and stored at 4°C in the dark until analysis (within one month). Total abundance of *Pseudo-nitzschia sp.* (cell $L^{-1}$) was determined by inverted light microscopy according to the Utermohl method (Uthermohl, 1958).

## II.4. Statistical analysis

Since environmental parameters, biological and histological data considered some experiences did not comply with the parametric assumption of normality and variance equality, non−parametric Kruskall−Wallis test ($n > 2$) followed by the post hoc Dunn test (joint ranking test) were used for pair wise comparisons as an alternative to ANOVA. These non-parametric tests were also used to analyse differences in total metals and PAHs concentrations in sediment and bioaccumulation in fish *in situ* study.

To test the null hypothesis of no significant differences of biological data *in situ* study and of molecular biomarker responses in microcosm study, one-way ANOVA, followed by post-hic Tukey tests were performed. Normality and homoscedasticity were tested using, respectively, Kolmogorov-Smirnov (using Shapiro-Wilk and Lilliefors corrected tables) and Bartlett tests. An analysis of covariance (ANCOVA) was used to compare length-weight relationships among estuaries, *in situ* study.

Box-and-whisker plots were used to interpret the distribution of biological data among different conditions of experimental studies.

Principal components analysis (PCA) was used to graphically represent correlations between sediment contaminants, biomarkers and fish biological responses in the experience of sea bass juveniles with estuarine sediment contamination.

To test the null hypothesis of no significant difference of TEP concentrations and *P. pseudodelicatissima* abundances between *Phaeocyctis* and TEP and between two physiological states of *P. pseudodelicatissima,* Wilcoxon Mann-Whitney ($n = 2$) were performed.

A significance level of a minimum of 5 % was considered in all statistical analyses. The statistics were performed using XLSTAT software package (version 5.01).

# CHAPTER III

# POLLUTION IMPACT ON FISH

# CHAPTER III - 1

# RELATING BIOLOGICAL RESPONSES OF JUVENILE FLOUNDER TO ENVIRONMENTAL CHARACTERISTICS AND SEDIMENT CONTAMINATION OF ESTUARINE NURSERY AREAS

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**ABSTRACT**

Estuaries serve as nursery grounds for many marine fish species. However, these attractive ecosystems are strongly affected by numerous anthropogenic activities and, the degree of human-induced alterations on their ecological functions remains largely unknown. The aim of this study was to evaluate the effects of chemical contamination on the biological responses of juvenile flounders and to assess and compare the quality of four estuarine habitats (Canche, Authie, Somme and Seine). Otolith growth and condition indices (RNA:DNA ratio, length-weight relationship, Fulton's K condition index) were measured on 0-group juvenile European flounder. Environmental conditions such as hydrological parameters (temperature, salinity, dissolved oxygen, turbidity), food availability and heavy metals, PAHs, PCBs and organic matter in sediments were analysed and compared among the four estuaries. Fish were analysed for metal bioaccumulation measurement. The Seine estuary was the most polluted area (higher metals and PAHs contents in sediment) and showed the highest enrichment factor indicating a high anthropogenic pressure. Metal concentrations in juvenile flounders of the

Seine estuary were also significantly higher than in the less polluted estuaries. The abundance of macrobenthos (potential preys for juvenile) was higher in the Seine estuary confirming the important role played by this estuary as feeding grounds in the studied area. However fish growth and condition indices were significantly lower in individuals from the Seine compared to those of the Canche, Authie and Somme. No obvious effects of hydrological condition or food availability on the flounder biological responses were found in this study. The present study emphasized the negative impact of contaminants on growth and condition of juvenile flounder. As the Seine is the single large estuary of the Eastern Channel, these alterations could affect important fish resources depending on estuarine nursery grounds in this area. More extensive research is needed in order to evaluate how pollutants are detrimental to flounder populations and more generally to estuarine fishes and the nursery function of estuaries.

Keywords: Flounder, estuarine nursery, habitat quality, growth, condition, chemical contamination.

III.1.2. Introduction

During their life cycle, marine fish species are commonly dependent on a number of different habitats (Pihl et al., 2005). Among these habitats, the role of estuaries as nursery grounds have been demonstrated in many studies (Miller et al., 1984; Amara et al., 2000; Vasconcelos et al., 2010). Sometimes referred in the literature as "essential fish habitat", these habitats are thought to provide ecological advantages for juvenile fishes and host higher juvenile density than adjacent coastal habitats (Beck et al., 2001). However, these attractive ecosystems are strongly affected by numerous anthropogenic activities and, the degree of human-induced alterations on their ecological functions remains largely unknown. Consequently, habitat quality has become a major concern for ecologists and a growing number of studies focus on the relationship between environmental quality and fish health (e.g. Burke et al., 1993; Able et al., 1999; Gilliers et al., 2004; Vinagre et al., 2008b). Some studies have shown that alteration in habitat quality can have a large impact on growth rate, survival, and subsequent recruitment of marine species with estuarine - dependent early life history stages (Phelan et al., 2000; Stunz et al., 2002; Amara et al., 2007; Vasconcelos et al., 2009). The need to protect these essential habitats exists and hence makes it necessary to develop indicators to estimate their quality.

The use of fish as biological indicators of estuarine quality evaluation and assessments of human impacts has several key advantages (reviewed by Whitfield and Elliott, 2002). As part of the European Water Framework Directive (WFD – Directive 2000/60/EC, 2000), fish are used to establish the ecological quality status of transitional waters (Franco et al., 2008; Delpech et al., 2010). However, the influence of natural and anthropogenic stressors on the structure of estuarine fish communities, as characterized by guilds, has not yet been demonstrated (e.g. Cabral et al., 2001; Elliott et al., 2007). Estuarine fish assemblages are subject to a great environmental variability that largely depends on estuary size and both upstream fluvial and downstream marine influences (Nicolas et al., 2010). Several studies have failed to relate fish communities analyses with anthropogenic pressure (Elliott and Dewailly, 1995; Selleslagh et al., 2009). Another constraint in the development of fish-based indices is the absence of a type-specific reference (Southerland et al., 2007). Moreover, because estuarine biological communities are well-adapted to cope with high stress, it is difficult to quantify the effects of anthropogenic stress on these communities; this is called the estuarine quality paradox (Elliott and Quintino, 2007). For example for fish, estuaries are used

by numerous marine species despite being usually subject to strong anthropogenic disturbances.

Fish display close physiological relationships to their environment as ectothermic organisms. Thus, they are sensitive to environmental disruptions, and particularly to chemical stress. That is why other approaches such as analyses of fish biological responses to specific and multiple stressors have been extensively used to determine individual health and population status, and to assess habitat quality (Phelan et al., 2000; Alquezar et al., 2006; Fonseca et al., 2006; Costa et al., 2009a; Franco et al., 2010). In particular, examination of individual fish growth and condition is one method that has been successfully used to compare habitat quality among different juvenile nursery areas (e.g. Sogard, 1992; Meng et al., 2001; Gilliers et al., 2006; Amara et al., 2007). The use of growth and condition as indices of habitat quality is based on the assumption that larger, faster-growing fish are healthier and hence experienced more favourable environmental conditions than smaller, slower-growing fish.

The French coast of the eastern part of the English Channel is bordered by several megatidal estuaries. These estuaries function as vital nursery grounds for numerous commercially important marine fish species such as plaice, sole and sea bass (Selleslagh and Amara, 2008a). In contrast to the small estuaries of the areas (Canche, Authie and Somme) which are considered as clean (low domestic, agricultural and industrial effluents), the Seine estuary is the largest megatidal estuary in the English Channel, is heavily impacted by manmade modifications and is contaminated with a complex mixture of chemical compounds (Munschy et al., 1997).

The present study investigates the relationship between environmental chemical contamination and fish biological responses to assess differential estuarine habitat quality. Growth and condition indices (otolith growth, RNA:DNA ratio, Fulton's K condition index), feeding and metal bioaccumulation were measured on juvenile European flounder, *Platichthys flesus* collected in four estuaries (Canche, Authie, Somme and Seine). Environmental conditions such as hydrological parameters, food availability and metals, PAHs, PCBs and organic matter contents in sediment were analysed and compared among the four estuaries. The main objective was to examine the sensitivity of fish biological responses to chemical contamination and to further test their utility as a monitoring tool for fish habitat quality.

European flounder is a temperate flatfish species commonly found in shallow waters from the North Sea to the Mediterranean. Juveniles of this species concentrate in estuaries and are one of the most important components of the demersal fish assemblage in European estuarine waters. Like other flatfish that use nearshore habitats as nursery grounds, juvenile flounders are sensitive to the effects of pollution and other types of habitat degradation, since they feed on benthic organisms and live in close association with the bottom sediments where most of the chemical contaminants introduced into aquatic environments by human activities accumulate.

## III.1.3. Materials and Methods

### III.1.3.1. Study area and sampling

The study area was located along the French coast of the Eastern English Channel (Figure 23). Four estuarine nursery grounds (Canche, Authie, Somme and Seine) were investigated in terms of different anthropogenic activities. The Canche, Authie and Somme are small estuaries (6.2; 10.4 and 40.9 $km^2$, respectively) with small freshwater input (15.6; 10.8 and 35.4 $m^3 s^{-1}$ respectively).These estuaries are considered as little impacted systems (little urban development without industrialisation) (Courat et al., 2009). Beside this, the multi-polluted Seine estuary is the largest megatidal estuary in the English Channel (197 $km^2$ with a mean annual river discharge of 435 $m^3 s^{-1}$), with a population of 16 million inhabitants, it concentrates 40% of the economic activity of France and 50% of the river traffic. Thus, pollutant inputs are very important in the Seine estuary. In addition to the disturbances resulting from manmade modifications of the estuary, high levels of heavy metals, particularly cadmium and lead, make the Seine estuary one of the most contaminated in Europe (Chiffoleau, 2001; Dauvin, 2008).

Juvenile flounders were collected in spring (late May - June) 2008 in the oligo- and meso- haline zones. Sampling was performed using a 1.5 m beam trawl (5 mm mesh size in the cod end) towed by a zodiac against the current at 2 knots for 15 min. Upon collection, fish were sorted alive on board and transported (in an ice box) to the laboratory, where they were immediately stored at -20°C until analysis.

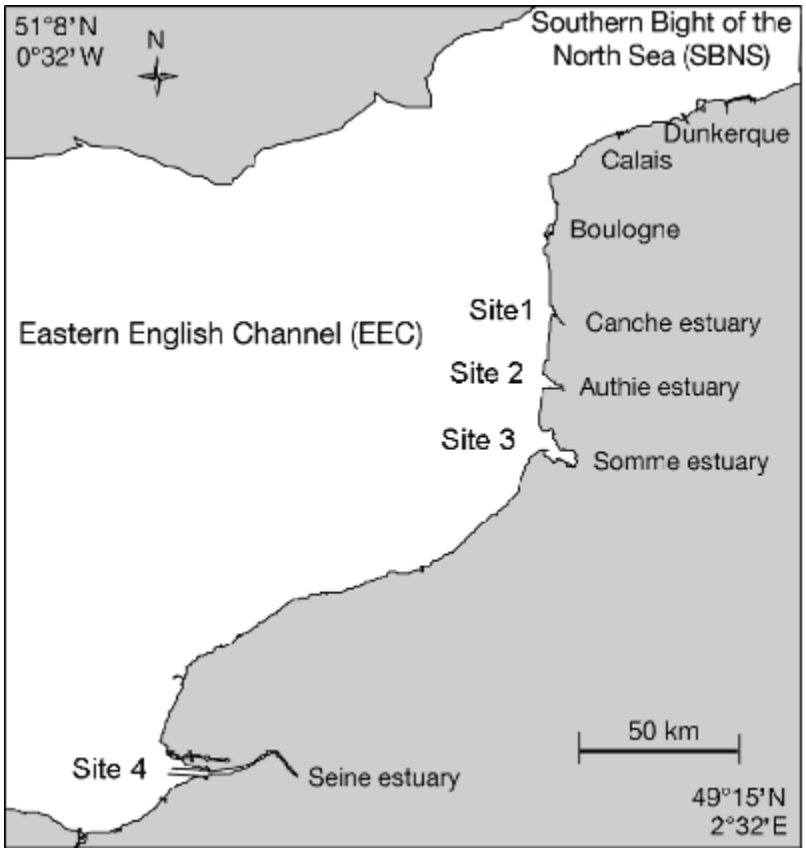


Figure 23. Locations of the four estuarine sites where 0-group flounder were collected during May-June 2008

### III.1.3.2. Environmental variables

Prior the fish sampling, water physicochemical parameters (temperature, salinity, dissolved oxygen, conductivity and turbidity) were measured using a Hanna HI 9828 multiprobes. At each station (3-4 stations in each estuary), three sediment samples were collected using a Van Veen grab (sampling an area of about 250 $cm^2$ to a sediment depth of ~10 cm) : one for macrobenthos analysis, one for granulometry and the third for organic matter (OM) content analysis. In order to measure metals, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), surface sediments were collected at low tide, at the same fish sampling station using a polypropylene syringe previously washed with diluted hydrochloric acid solution. Sediment samples were afterwards put in polyethylene bags and stored in an ice box. In the laboratory, the samples were kept at – 20 °C until further treatment and analysis.

Sediment samples for macrobenthos were washed and sieved through a 1 mm mesh in the laboratory. All organisms retained on the sieve were collected, stained with Rose Bengal and preserved in 5% formaldehyde buffer for subsequent identification. Macrobenthos samples were analysed up to species level using a binocular microscope. Those organisms, which cannot be identified to species level, were classified in the generic level or up to the lowest taxonomic level. Macrobenthic organisms were counted and their abundances (individuals.$m^{-2}$) and species occurrence (%) were calculated for each estuary. In addition, AFDW (Ash Free Dry Weight, $g.m^{-2}$) biomass values were then measured. For the Seine estuary, the data on macrobenthos abundance come from a database called Macrobenthos of the Bay and Estuary of Seine (MABES); available via the data administrator of the GIP Seine Aval: nbacq@seine-aval.fr). From this database, we chose five stations sampled in spring 2008 near our flounder sampling.

The granulometry of the upper 15 cm layer of an humid aliquot of sediment sample was analysed using a laser Beckman-Coulter LS 230. A classification was established using the proportion of clay (< 4 µm), fine silt (4 − 20 µm), coarse silt (20 − 50 µm), fine sand (50 − 200 µm), medium sand (200 − 500 µm) and coarse sand (500 − 2000 µm). For the measurement of total organic matter (TOM, mg $g^{-1}$), sediment samples (~ 2 g) were dried at 60°C for 72h and subsequently burned at 500°C for 6h. The TOM was calculated by the difference between total dry-weight and ash-weight of sediments (Luczak et al., 1997). In addition, the total organic carbon, (TOC) and total organic nitrogen (TON) contents were determined using a CHNS analyzer (NA 2100, CE instruments).

### III.1.3.3. Sediment contaminant analysis

To avoid contamination, all chemical reagents used for the analysis were chosen of Suprapur quality (Merck) and all materials were intensively cleaned with acid and rinsed with ultra clean water (Milli-Q) before use. Sediments were dried at 40°C to constant weight and were ground to powder using an agate mortar and pestle.

#### III.1.3.3.1. Metal analysis

For total metal determination, about 0.3 g ground sediments were digested with HF at 110 °C for 48 h followed by a mixture of concentrated acids HCl:$HNO_3$ (3:1, v:v,) at 120 °C

for 24 h, operation renewed once. Metals associated with the reactive fractions of sediment, considered as bioavailable fractions, were estimated using the method of Huerta-Diaz and Morse (1990). The reactive fraction such as metals extracted by HCl 1 M comprises metal exchangeable and linked to carbonates and partially to oxy-hydroxides of Fe-Mn. About 0.5 g of sediment was leached during 24 h with 20 mL of 1 M HCl. The total and extractable heavy metals were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES, VARIAN Vista Pro, axial view). For quality assurance, reagents blanks, sample replicates and standard reference materials (HISS-1, MESS-3 and PACS-2, National Research Council Canada) were used to assess the accuracy and precision of the analysis. In all cases, the recovery efficiency was better than 85% for the total digestion of standard reference materials. Total Hg was measured in dry and ground sediment samples (without any pre-treatment) by means of atomic absorption spectroscopy (AAS) using an AMA 254 solid phase Hg-Analyzer (Altec Ltd., Prague, Czech Republic) (Ouddane et al., 2008). Mean recovery for total Hg was between 80 and 100% for certified estuarine sediment IAEA-405 (IAEA, Vienna, Austria).

### III.1.3.3.2. PAHs and PCBs analysis

The persistent organic pollutants, including PAHs (EPA's 16 priority PAHs) and PCBs (7 congeners) were analysed. Organic compounds were extracted from 2 g dried sediment by microwave (120 °C for 15 min, 1200 W) assisted extraction with 40 mL of a mixture of acetone and hexane (1:1, v:v). The solvent was evaporated under a stream of nitrogen in a TurboVap and then concentrated to 1 mL of hexane. Simultaneous determination of PAHs and PCBs was performed on a gas chromatography-mass spectrometry (GC-MS, VARIAN, CP 3800 – 1200 MS TQ). A ZB-MultiResidue column (30 m, 0.25 mm, 0.25 µm) were used (Phenomenex). Identification of PAH compounds and PCB congeners was based on the comparison of their GC-retention times and their mass spectrum, with appropriate individual standards.

### III.1.3.4. Fish metal analysis

For metal analysis, 10 juvenile flounders of each estuary were unfrozen at room temperature and their total length and weight recorded (44.5 ± 6.2 mm; 927.3 ± 387.5 mg).

Subsequently, whole-body fish were lyophilised during 48h. Before acid digestion, an agate was employed to grind and to homogenise the dry tissue samples. Approximately 0.5 g of aliquots was digested in Teflon beakers for 12h at room temperature and then 4h at 100°C in hot plate with ~ 4ml nitric acid. After that, the remaining digested solution was diluted with Milli-Q for all elements (Henry et al., 2004). Concentrations of Cd, Cr, Cu, Mn, Ni, Pb, V, Zn, As and Se were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Varian 820-MS). ICP apparatus were calibrated using standard solutions and the accuracy of the applied analytical procedure was tested using certified reference materials (DORM-3, fish protein) provided by the National Research Council of Canada.

### III.1.3.5. Biological analysis

#### III.1.3.5.1. Growth and condition indices

At the laboratory, 0-group juvenile flounders were defrosted, measured for SL (standard length) and TL (total length) to the nearest 0.1 mm and weighed (wet weight) to the nearest 0.001 g. The fish length-weight relationships were analysed for each estuary (n = 507, 0-group flounder).

Otoliths were analysed in order to estimate the recent growth index and to explore the otolith–fish size relationship as a tool for evaluating growth differences between estuaries (as suggested by Secor and Dean, 1989). In fact, results from several studies (Secor and Dean, 1989, Wright et al., 1990; Hare and Cowen, 1995) suggest that slower-growing fish may in fact have larger otoliths than faster-growing fish of similar sizes or ages. Sagittae were removed, cleaned and the right and left sagitta separated and photographed to measure the diameter and perimeter of each otolith. Since there was no significant difference in the parameters measured between the two sagittae (ANOVA, $p>0.05$), only the right sagitta was used in the analyses. The sagittae were mounted on microscopical slides, sulcus up, with cyanocrilate glue and polished with grinding paper of decreasing grit sizes (5 to 0.1 µm) until increments at the outer edge were visible. The recent growth index (RG; µm) was determined by measuring the width of the peripheral daily increments of the otoliths. Because there was a significant relationship between sagittal diameter and fish length (Figure 24), we used daily otolith increments from the previous 10 days before capture as an indicator of recent growth (mean distance between the margin of the otolith back to the 10th ring). All the measurements

were done along the same otolith axis (anteroposterior) using an Image Analysis System (TNPC, 5.0; © NEOSIS).

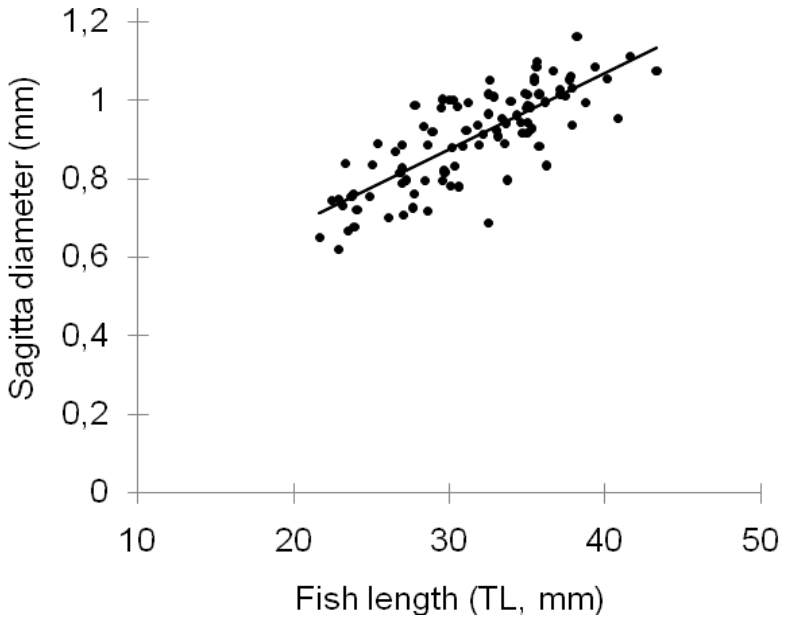


Figure 24. The relationship between fish TL (mm) and the sagittal otolith diameter (µm) in 0-group flounder. Regression model: diameter = 0.0196 + 0.286 (TL); n = 94. $R^2 = 0.63$, $p < 0.001$

We estimated two condition indices: the RNA-DNA ratio as an indicator of growth and nutritional status and the Fulton's K condition index as an indicator of the general well being. This latter morphometric index assumes that heavier fish for a given length are in better condition. Individual condition factor (K) was determined from morphometric data, according to the formula $K = (100 \times W) / TL^3$, where W is the body mass (mg) and TL is the total length (mm).

The procedure used to determine RNA and DNA concentrations in individual fish is based on the Clemmesen method (1988). However, heads and fins were discarded before analysing fish and guts were excised to ensure that gut contents did not contribute to RNA–DNA ratio. Fish muscle sample (0.05 g) was homogenized in ice–cold Tris–EDTA buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 8.0) using an Ultraturrax and subsequently transferring to a mixture of Tris–EDTA buffer, proteinase–K (pro–K) and sodium dodecyl

sulfate (SDS). Nucleic acids were extracted by purification step involving phenol−chloroformisoamylalcohol (Amara et al., 2009). The quantity of RNA and DNA was determined by the fluorescence-photometric technique using a specific nucleic acid fluorescent dye−ethidium bromide (Sigma−Aldrich Chemicals, France). The fluorescence due to total RNA was calculated as the difference between total fluorescence (RNA and DNA) and the fluorescence after RNAase treatment, which is assumedly to be due to the presence of only DNA. Salmon sperm DNA (Sigma−Aldrich Chemicals, France) and yeast type III RNA (Sigma−Aldrich Chemicals, France) were used as standards. RNA and DNA contents are both expressed as µg per µL.

### III.1.3.6. Feeding analysis

The gut (stomach + intestine) content of 180 juvenile flounders were dissected and analysed under a stereomicroscope. The main prey categories were identified and counted and the percentage of empty gut calculated.

### III.1.3.6. Statistical analysis

Differences in environmental parameters were analysed with non parametric Kruskal – Wallis test and the Dunn test (joint ranking test) for post hoc pairwaise comparisons among estuaries. These non-parametric tests were also used to analyse differences in heavy metals, PAHs, PCBs and the bioaccumulation in fish. Biological data were tested to assess significant differences among estuaries using one-way ANOVA, followed by post-hoc Tukey tests. Normality and homoscedasticity were tested using, respectively, Kolmogorov-Smirnov (using Lilliefors corrected tables) and Bartlett tests. Length-weight relationships were compared among estuaries using an analysis of covariance (ANCOVA).The statistics were performed using XLSTAT software package (version 2009.5.01) and a 0.05 (or lower) significance level was considered in all test procedures.

## III.1.4. Results

### III.1.4.1. Environmental variables

The Seine estuary differs from the other estuaries by its significantly higher water turbidity, sediment total organic matter (TOM), carbone (TOC) and nitrogen (TON) content. The sediment of the Canche, Authie and Somme estuaries was composed mainly by sand (fine, medium, coarse) whereas mud (clay, fine and coarse silt) was the main component of sediment from the Seine (71.28%) (Table 1). The Seine estuary is also characterised by higher macrobenthos abundance (1417 ± 976.0 ind $m^{-2}$). The dominant macrobenthic species was different among the estuaries: oligochaetes, polychaetes (*Hediste diversicolor*) and amphipods (*Bathyporeia sarsi* and *Gammarus duebeni*) were dominant in the Canche and Authie, whereas amphipods (*Corophium volutator*), polychaetes (*Hediste diversicolor*) and lamellibranch (*Macoma balthica, Scrobicularia plana*) dominated the macrobenthos of the Seine estuary (Table 2).

Total mean sediment concentrations (Cd, Cr, Cu, Hg, Ni, Pb, V, and Zn) were significantly higher (KW, $p<0.05$) in the Seine estuary (310.4 ± 18.0 mg $kg^{-1}$) compared to the Canche (37.8 ± 4.2 mg $kg^{-1}$), Authie (73.0 ± 4.7 mg $kg^{-1}$) and Somme (24.3 ± 2.4 mg $kg^{-1}$) estuaries. For each element analysed, the concentration was always significantly higher in the Seine estuary (KW, $p<0.05$) (Table 3). The sum of 16 PAHs measured in sediment was also significantly higher in the Seine estuary (KW, $p<0.05$). PCBs concentrations were not listed because they remain lower than the limit detection of the method used (0.01 mg kg-$^{1}$ to determine one congener content).

Table 1. Mean (±SD) values of abiotic and biotic parameters of the Canche, Authie, Somme and Seine estuaries. AFDW : Ash Free Dry Weight; TOM : total organic matter; TON : total organic nitrogen ; TOC : total organic carbon

| | | | | | | Macrobenthos | | | Sediment | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sampling Dates | Temperature (°C) | Salinity | Oxygen (mg $l^{-1}$) | Turbidity (NTU) | Abundance (ind $m^{-2}$) | AFDW (g $m^{-2}$) | TOM (mg $g^{-1}$) | TON (mg $g^{-1}$) | TOC (mg $g^{-1}$) | clay % | fine silt % | coarse silt % | fine sand % | medium sand % | coarse sand % |
| Canche | 31.05.2008 | 13.6 ± 0.1 | 0.1 ± 0.1 | 9.2 ± 0.1 | 16.7 ± 4.8 | 160 ± 40.0 | 0.1 0.1 | 9.2 ± 2.3 | 0.1 ± 0.1 | 1.3 ± 0.3 | 0.3 ± 0.2 | 0.6 ± 0.3 | 0.8 ± 0.2 | 48.4 ± 14.5 | 45.2 ± 11.6 | 4.6 ± 2.2 |
| Authie | 02.06.2008 | 14.1 ± 1.1 | 4.7 ± 4.1 | 9.6 ± 0.5 | 13.3 ± 3.6 | 860 ± 814.9 | 0.3 ± 0.3 | 18.5 ± 3.5 | 0.6 ± 0.2 | 4.9 ± 1.6 | 6.1 ± 1.8 | 15.7 ± 5.9 | 20.2 ± 9.6 | 51.3 ± 8.1 | 6.6 ± 9.1 | 0.1 ± 0.2 |
| Somme | 01.06.2008 | 17.0 ± 0.6 | 16.3 ± 12.6 | 11.6 ± 1.4 | 19.0 ± 4.0 | 680 ± 792.0 | 0.1 ± 0.1 | 9.5 ± 2.3 | 0.0 ± 0.0 | 2.2 ± 1.7 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.1 ± 0.1 | 38.9 ± 0.6 | 59.7 ± 0.5 | 1.2 ± 0.1 |
| Seine | 07.06.2008 | 17.6 ± 0.3 | 12.8 ± 5.2 | 8.9 ± 4.0 | 87.5 ± 3.5 | 1417 ± 976.0 | 0.5 ± 0.6 | 61.6 ± 5.6 | 2.4 ± 0.2 | 16.5 ± 1.0 | 8.3 ± 1.0 | 28.7 ± 4.1 | 34.3 ± 3.2 | 28.7 ± 2.6 | 0.0 | 0.0 |

Table 2. Occurrence (%) and abundance (ind $m^{-2}$) of macrobenthic species collected in the four estuaries in June 2008

| Group | Species | Canche | | Authie | | Somme | | Seine | |
|---|---|---|---|---|---|---|---|---|---|
| | | Occurrence (%) | Abundance (ind. $m^{-2}$) | Occurrence (%) | Abundance (ind. $m^{-2}$) | Occurrence (%) | Abundance (ind. $m^{-2}$) | Occurrence (%) | Abundance (ind. $m^{-2}$) |
| **Polychaetes** | *Hediste diversicolor* | 0.33 | 26.7 | 1 | 230.0 | 0 | 0.0 | 1 | 172.0 |
| **Oligochaetes** | *Oligochaeta sp.* | 0.33 | 40.0 | 0.75 | 360.0 | 0 | 0.0 | 0.33 | 17.0 |
| **Amphipods** | *Bathyporeia sarsi* | 0.33 | 40.0 | 0.25 | 20.0 | 1 | 680.0 | 0 | 0.0 |
| | *Gammarus duebeni* | 0.33 | 13.3 | 0.25 | 230.0 | 0 | 0.0 | 0 | 0.0 |
| | *Gammaropsis maculata* | 0 | 0.0 | 0.25 | 10.0 | 0 | 0.0 | 0 | 0.0 |
| | *Corophium volutator* | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0.67 | 1100.0 |
| **Lamellibranch** | *Macoma balthica* | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0.33 | 117.0 |
| | *Scrobicularia plana* | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0.33 | 11.0 |
| **Mysids** | *Neomysis integer* | 0.33 | 26.7 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 |
| **Egg** | *Egg* | 0.33 | 13.3 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 |
| **Fish** | *fish larvae* | 0 | 0.0 | 0.25 | 10.0 | 0 | 0.0 | 0 | 0.0 |
| **Total** | 11 | 6 | | 6 | | 1 | | 5 | |

Table 3. Mean (±SD) sediment heavy metal and ΣPAH concentrations (mg $kg^{-1}$ dry weight) in the Canche, Authie, Somme and Seine estuaries *n.d.: no detected for PAH < 0.05

| | Cd | Cu | Cr | Hg | Mn | Ni | Pb | V | Zn | Al | Fe | ∑ PAHs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Canche | 0.11 ± 0.02 | 1.49 ± 0.18 | 8.58 ± 0.93 | 0.002 ± 0.003 | 137 ± 4 | 2.04 ± 0.15 | 6.14 ± 0.60 | 8.27 ± 1.95 | 11.2 ± 1.7 | 9834 ± 953 | 5040 ± 55 | 0.18±0.04 |
| Authie | 0.36 ± 0.05 | 3.66 ± 0.48 | 19.4 ± 2.0 | 0.019 ± 0.012 | 191 ± 2 | 4.02 ± 0.24 | 8.76 ± 0.67 | 16.42 ± 3.08 | 20.3 ± 2.2 | 13 093 ± 1256 | 7879 ± 866 | 0.08 ± 0.02 |
| Somme | < 0.1 | 1.00 ± 0.09 | 5.56 ± 1.96 | 0.002 ± 0.001 | 59.3 ± 17.1 | 1.28 ± 0.16 | 4.66 ± 0.46 | 5.23 ± 0.14 | 6.60 ± 0.54 | 8254 ± 237 | 3195 ± 640 | n.d .* |
| Seine | 0.90 ± 0.14 | 25.27 ± 3.26 | 64.0 ± 4.6 | 0.361 ± 0.032 | 476 ± 24 | 13.5 ± 1.2 | 35.0 ± 4.3 | 48.9 ± 3.8 | 123 ± 3 | 30 502 ± 859 | 20 099 ± 1032 | 1.00 ± 0.03 |

In all estuaries, the same tendency of the proportion of bioavailability of metals in sediments was observed (Figure 25). Some elements such as Cu, Mn, Pb and Zn showed strong affinity with the acid-soluble fraction, which represents more than 60 % while Cr, Ni and V remained lower than 30 % among the estuaries.

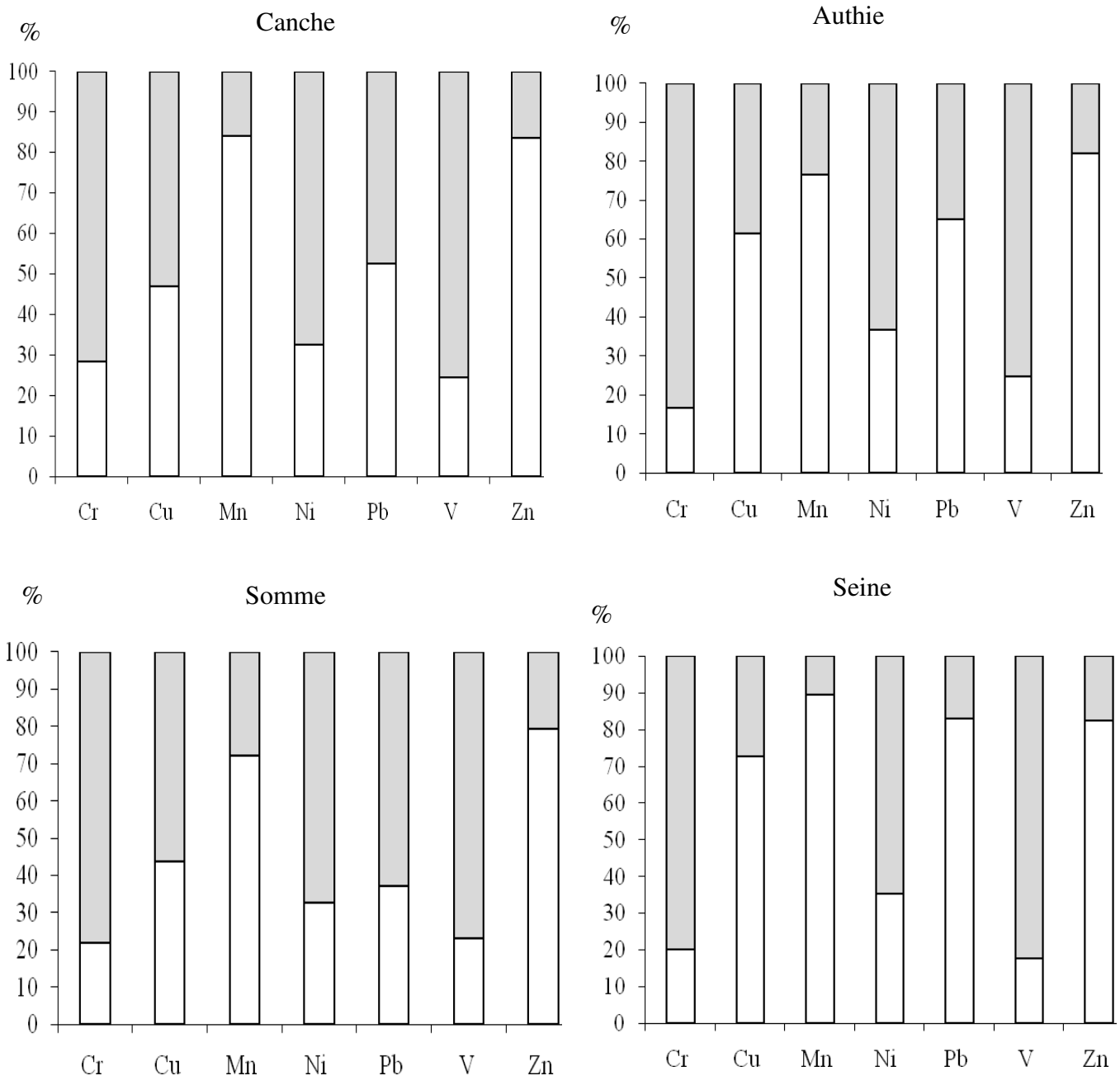


Figure 25. Distribution (%) of metals in the two sedimentary fractions (□ bioavailable; ■ non bioavailable) for the four estuaries (Canche, Authie, Somme, and Seine)

The extent of sediment contamination was determined using the enrichment factor (EF) (Salomons and Forstner, 1984). Commonly, normalization of the heavy metals to a conservative element such as Al is employed as an index to evaluate anthropogenic influences to the sediments. The EF is defined as:

EF = $(X/Al)_{sample} / (X/Al)_{background;}$ where $(X/Al)_{sample}$ is the metal to Al ratio in the samples of interest; $(X/Al)_{background}$ is the natural background value of metal to Al ratio. EF values were interpreted as suggested by Birth (2003) for the metals studied with respected to upper crust average (Taylor and McLennan, 1995). EF < 1 indicates no enrichment, EF < 3 is minor enrichment, EF = 3–5 is moderate enrichment, EF = 5–10 is moderately severe enrichment, EF = 10–25 is severe enrichment, EF = 25–50 is very severe enrichment and EF > 50 is extremely severe enrichment. Table 4 presents the mean EF values of the metal studied with respect to crust average. The Somme estuary showed no (Cu, Ni, V, Zn) and minor (Cr, Mn, Pb) metal enrichment level. Therefore, all metals (except Pb) were less than 2, showing a minor anthropogenic impact on the heavy metals concentration levels in this estuary. As a result, these metals pollution should not be currently a major concern for this estuary. The Canche showed minor (Cr, Mn, Pb, V, Zn) and moderate enrichment with the highest EF value of Cd. The mean EF values were greater than 2 for Cd, Cr and Pb which can be explained by various degrees of metal enrichment. However, this estuary can also be considered as no polluted estuary. Both the Authie and the Seine estuaries showed EF values of severe anthropogenic enrichment of Cd while other elements presented minor and moderate enrichment. Hence, the metals contained in the sediment from the sampling zones in the Seine estuary might in major part come from non-crustal materials or non-natural weathering processes; it contains the highest EF values for all elements (Seine > Authie > Canche > Somme).

Table 4. Mean metal enrichment factor (EF) in sediment samples of the Canche, Authie, Somme and Seine estuaries

| Metal | Canche | Authie | Somme | Seine |
|---|---|---|---|---|
| Cd | 9.6 | 23.0 | - | 24.2 |
| Cr | 2.0 | 3.4 | 1.5 | 4.8 |
| Cu | 0.5 | 0.9 | 0.4 | 2.7 |
| Mn | 1.9 | 2.0 | 1.0 | 2.1 |
| Ni | 0.8 | 1.2 | 0.6 | 1.8 |
| Pb | 2.5 | 2.7 | 2.3 | 4.6 |
| V | 1.1 | 1.7 | 0.8 | 2.1 |
| Zn | 1.3 | 1.8 | 0.9 | 4.6 |

The total mean concentrations of heavy metals in fish (Cd, Cr, Cu, Ni, Pb, As, Se and V) were significantly higher (KW, $p<0.05$) in the Seine (17.9 ± 5.1 $mg.kg^{-1}$) compared to the Canche (9.5 ± 0.9 $mg.kg^{-1}$) and Authie (10.5 ± 1.3 $mg.kg^{-1}$) estuaries. In the Somme estuary, the mean total concentration of selected metals (13.8 ± 0.6 $mg.kg^{-1}$) was lower than in the Seine but not significantly different. The concentrations of heavy metals in fish (Cu, Ni, Pb, Se, V) were significantly higher (KW, $p<0.05$) in the Seine except for Mn and As which displayed the highest value in the Authie and the Somme, respectively (Table 5).

Table 5. Mean (±SD) metal concentrations (mg $kg^{-1}$ dry weight) in 0-group flounder from the Canche, Authie, Somme and Seine estuaries. (n = 10 fish / estuary)

| | Cd | Cu | Cr | Mn | Ni | Pb | V | Zn | As | Se |
|---|---|---|---|---|---|---|---|---|---|---|
| Canche | 0.02 ± 0.01 | 2.91 ± 0.24 | 0.29 ± 0.07 | 23.6 ± 1.4 | 0.11 ± 0.03 | 0.19 ± 0.05 | 0.30 ± 0.05 | 118 ± 23 | 3.81 ± 0.33 | 1.88 ± 0.32 |
| Authie | 0.04 ± 0.01 | 3.94 ± 0.90 | 0.23 ± 0.07 | 28.7 ± 2.0 | 0.19 ± 0.12 | 0.11 ± 0.02 | 0.21 ± 0.01 | 121 ± 12 | 4.32 ± 0.39 | 1.44 ± 0.14 |
| Somme | < 0.01 | 3.58 ± 0.56 | 0.33 ± 0.04 | 10.4 ± 0.6 | 0.21 ± 0.08 | 0.18 ± 0.01 | 0.22 ± 0.02 | 107 ± 8 | 7.29 ± 0.43 | 1.96 ± 0.11 |
| Seine | 0.04 ± 0.1 | 7.68 ± 0.12 | 0.53 ± 0.25 | 19.9 ± 1.8 | 0.52 ± 0.12 | 0.83 ± 0.08 | 0.69 ± 0.14 | 137 ± 9 | 1.86 ± 0.60 | 2.64 ± 0.09 |

### III.1.4.1. Fish biological responses

The size and length of 507 0-group flounder were measured (234 in the Canche, 215 in the Authie, and 60 in the Seine). The length-weight relationship of flounder from the Seine estuary was significantly lower compared to the Canche and Authie estuaries (ANCOVA, $p<0.05$) (Figure 26). Growth and condition indices were measured on 30 individuals in each estuary except for the Somme estuary were only 5 individuals were analysed (Table 6). The mean total length and weight of the flounders analysed were, respectively, 31.96 ± 4.99 mm and 359.1 ± 179.7 mg. There was no significant difference for size and weight of the individuals analysed among the four estuary (ANOVA, $p>0.05$).

The perimeter of the otolith (sagitta) standardised by the fish total length (TL) showed significant variation among estuaries (Table 6). This otolith index was significantly lower in the Seine estuary compared to the three other estuaries. The Canche has the significantly higher otolith index value. The mean recent growth index (mean otolith increment widths for

the last 10 days) of flounder analysed was 6.03 ± 1.50 µm. Individuals from the Seine had slower RG (5.34 ± 1.44 µm). RG showed significant differences among the Authie (6.63 ± 1.51 µm) and the Seine and did not vary significantly among flounder from the Canche and Somme estuaries (6.19 ± 1.45 µm and 5.73 ± 0.32 µm, respectively) (Table 6).

The Fulton's K of the individual 0-group flounder analysed varied between 0.24 and 2.74 (mean value = 1.14 ± 0.55 mg $mm^{-3}$). Individuals from the Seine have a significantly (ANOVA, $p<0.05$) lower K (0.96 ± 0.09 mg $mm^{-3}$) compared to the three others estuaries. The individuals from the Canche have the significantly higher K value (1.05 ± 0.11 mg $mm^{-3}$) (ANOVA, $p<0.05$).

As the previous indices, RNA concentration also differed significantly among estuaries (ANOVA, $p<0.05$). Individuals from the Seine estuary had a significantly (ANOVA, $p<0.05$) lower RNA concentration (119.83 ± 64.31 µg/µL). The RNA-DNA ratio also differed significantly among estuaries (ANOVA, $p <0.05$). It varied from 0.16 to 6.91 (mean value = 2.07 ± 0.91). Individuals from the Seine estuary had a significantly (ANOVA, $p<0.05$) lower RNA-DNA ratio (1.73 ± 0.73) compared to those of Canche (2.45 ± 1.07).

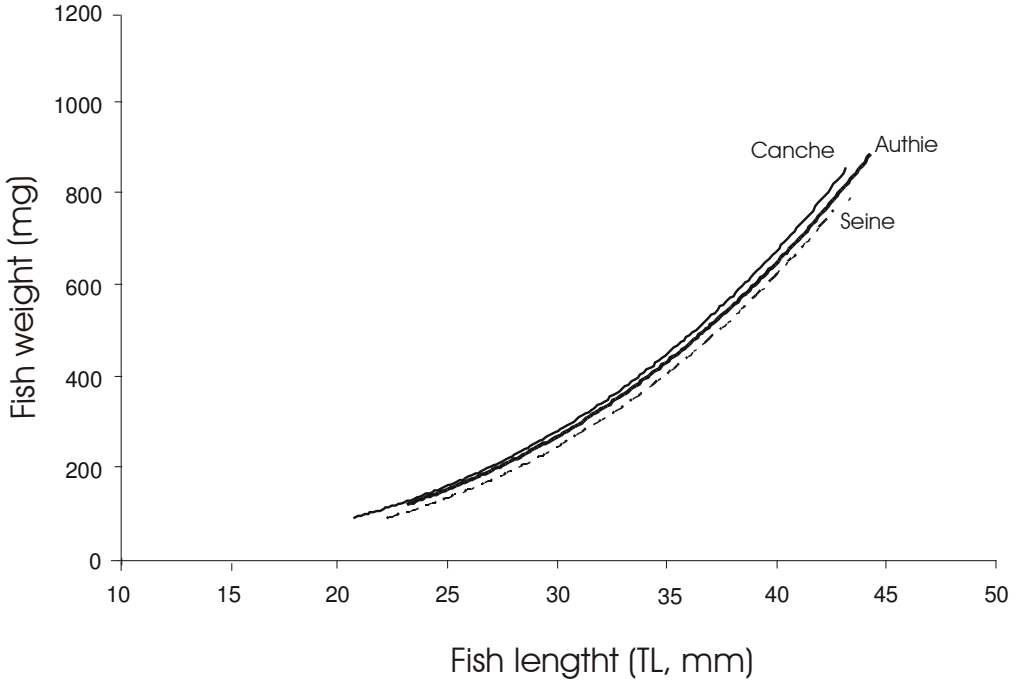


Figure 26. Length-weight relationships of 0-group flounder in the Canche (n = 234; $W = 0.0085\ TL^{3.06}$, $R^2 = 0.95$), Authie (n = 215; $W = 0.0089\ TL^{3.04}$, $R^2 = 0.94$) and Seine (n = 60; $W = 0.0054\ TL^{3.16}$, $R^2 = 0.97$)

Table 6. 0-group flounder total length (mm), weight (mg), RNA concentration (µg. µl$^{-1}$), RNA-DNA ratio, Fulton's K condition index (mg.mm$^{-3}$), otolith recent growth (RG, µm) and otolith perimeter: fish length ratio

| Estuary | N | Total length (TL, mm) | Total weight (mg) | K (mg mm$^{-3}$) | RNA:DNA | RNA (µg/µL) | RG (µm) | Otolith perimeter/TL |
|---|---|---|---|---|---|---|---|---|
| Canche | 30 | 30.24 ± 4.68 | 314.25 ± 157.98 | 1.05 ± 0.11 | 2.45 ± 1.07 | 261.02 ± 44.27 | 6.20 ± 1.45 | 0.073 ± 0.006 |
| Authie | 29 | 31.30 ± 4.61 | 333.67 ± 166.76 | 1.02 ± 0.11 | 1.98 ± 0.76 | 188.58 ± 86.96 | 6.63 ± 1.51 | 0.077 ± 0.004 |
| Somme | 5 | 34.63 ± 5.46 | 453.58 ± 210.34 | 1.0 ± 0.08 | - | - | 5.73 ± 0.32 | 0.078 ± 0.006 |
| Seine | 30 | 31.68 ± 5.22 | 334.91 ± 183.74 | 0.96 ± 0.09 | 1.73 ± 0.73 | 119.83 ± 64.31 | 5.34 ± 1.44 | 0.086 ± 0.005 |

0-group flounders feed on a large category of preys but their main prey is copepods and amphipods (Figure 27). Although not in the same proportion, they feed on the same preys in the four estuaries (mainly copepods, amphipods, some isopods, polychaetes (*Nereis diversicolor*) and insects' larvae (*chironomid larvae*).The percentage of empty gut was the highest in the Somme (60.0%) and the Seine (50.9%) compared to the Authie (18.3%) and the Canche (38.3%) estuaries.

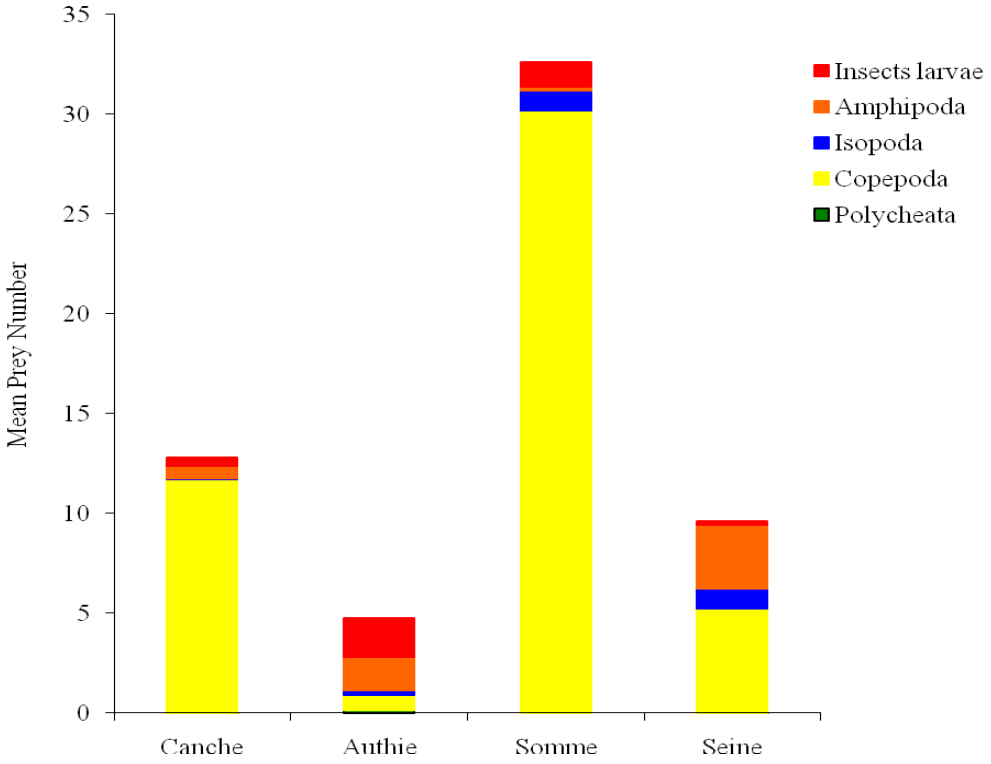


Figure 27. Composition of the diet (number of major prey taxa) of 0-group flounder sampled in the four estuaries

III.1.5. Discussion

The main objective of this study was to evaluate if fish condition and growth indices could be used as proxy to assess estuarine habitat quality. The use of growth and condition as indices of habitat quality is based on the assumption that larger, faster-growing fish are healthier and hence experienced more favourable environmental conditions than smaller, slower-growing fish. We found significant differences among the biological indicators measured on 0-group juvenile flounders collected from estuaries with different degrees of

anthropogenic disturbance. Juvenile flounders grew slower and had lower condition indices in the Seine estuary, which indicate habitat of inferior quality for juvenile fishes.

Estuarine organisms are exposed to a wide range of stressors due to the highly complex and variable nature of estuarine systems. Selection and development of biological indicators that can distinguish anthropogenic effects from natural variability is at the heart of habitat quality assessment (Adams, 2002). When a difference in habitat quality is demonstrated, it is necessary to show that the observed differences are related to human-induced changes and not to natural factors. A comparison of habitats exposed to high anthropogenic pressures to less polluted ones provides a way to identify useful biological indicators. The main environmental differences recorded among the four studied estuaries concerns macrobenthos abundances, sediment grain size characteristic, total organic matter (TOM), carbone (TOC) and nitrogen (TON) content and sediment chemical contaminants.

Fish growth and condition may be affected by numerous environmental factors. Food availability is considered a critical limiting factor in flatfish growth and may be an element in estuarine and habitat-related differences in growth or condition (Sogard, 1992; Gibson, 1994). Estuarine habitats generally offer high densities of prey and other food not encountered in marine areas (McLusky and Elliott, 2004). It is one of the reasons why many juvenile marine fish use estuaries as nursery ground. For example, along the Eastern English Channel coast the abundance of the benthic macrofauna (preys for juvenile fish) inside the estuaries is two to four time higher than in the adjacent shallow marine coastal areas (Selleslagh et al., 2009). Small juvenile flounders feed on a large category of preys. Previous studies have found juvenile flounders to be opportunistic generalists, feeding on a variety of polychaetes, crustaceans and amphipods (Vinagre et al., 2008a). More generally, it is recognized that juvenile flatfish consume the most abundant food resources in a generalistic and opportunistic manner (e.g. Beyst et al., 1999; Amara et al., 2001), which strongly reduces their food limitation in highly productive systems such as estuarine habitats. Numbers of studies have failed to find a robust link between prey abundance and growth in juvenile flatfish (Sogard, 1992; Manderson et al., 2002; Amara, 2003). The Seine estuary supports high benthic biomass (Dauvin, 2008; this study) and prey availability could not explain the observed differences in growth and condition of flounder. A possible cause of the lower gut fullness values in the Seine estuary found in this study is the reduced foraging efficiency that juvenile European flounder may experience in this estuary where the physical or structural feeding environment is suboptimal (higher turbidity, muddy sediment). For example, it has been

showed that the predation efficiency of European flounder was reduced with increasing habitat complexity both in wild (Tarpgaard et al., 2005) and in laboratory experiment with filamentous drift algae (Aarnio and Mattila, 2000). This species is known to tolerate large fluctuations in abiotic factors such as temperature and salinity (Lundgreen and Jensen, 2000). No obvious effects of hydrological factors on growth were found in this study. Temperature (Fonds et al., 1992) or dissolved oxygen (Tallqvist et al., 1999) was within the range where food consumption and growth rates are high for juvenile European flounder. All this suggests that lower growth and condition indices of 0-group flounder in the Seine estuary may probably be due to other factors directly related to human activities such as chemical contamination.

The evaluation of sediment quality is an important part of assessing the quality of estuarine environments. We found that the measured sediment contamination reflects the estuarine anthropogenic pressure. The Seine estuary is characterised by high concentrations of a variety of contaminants, including metals and PAHs. This estuary was described as one of the most contaminated in Europe, particularly for cadmium and lead concentrations (Chiffoleau, 2001). Although the metal contamination levels are currently 2 to 6 times lower in the 2000's than they were in the 1950's (Meybeck et al., 2007). For each element analysed in the present study, the concentration was always significantly higher in the Seine estuary than in the Canche, Authie or Somme estuaries. Our data demonstrate that the sediment from the sampling zones in the Seine estuary have the highest enrichment factor (EF) values particularly for Cd suggesting an important metal accumulation of anthropogenic origin. In the Seine, the contaminant input comes primarily from the upper part of the estuary due to the industrial activities and high urbanization of the Paris region (12 million inhabitants) (Dauvin, 2008). A comparison of Cu, Pb, and Zn concentrations in the biological compartments (benthic and suprabenthic species) of the Seine estuary with those found in the same species collected at non-contaminated sites demonstrated the high level of contamination of this estuary (Miramand et al., 2001). In the present study, we found that the concentrations of heavy metals in fish were generally significantly higher in the Seine.

Pollutants can induce various biological responses in fish, affecting the organisms from the biochemical to the population-community levels (Adams, 2002). High habitats quality are assumed to be those where growth, survival and future reproductive potential are optimized (Gibson, 1994). Fish growth rates and condition have been widely observed to relate inversely to exposure of the fish to contaminants (Rowe, 2003; Alquezar et al., 2006;

Amara et al., 2007). Burke et al. (1993) showed that the condition and growth of Atlantic croaker were depressed along an estuarine pollution gradient. In a study carried out in the Eastern English Channel, Amara et al. (2007) showed that shallow coastal nurseries with highest sediment chemical contaminants had the lowest habitat quality for juvenile flatfish. For example, exposure to high concentrations of copper in the water had direct physiological costs for juvenile Senegalese sole, expressed as decreased growth and lower physiological condition (RNA:DNA values), even under optimal feeding conditions (Fonseca et al., 2009). Many investigations on biological responses of flounder populations to contamination indicated a general decreased of the relative fecundity, the growth rate and condition factor in contaminated estuaries (Laroche et al., 2002; Marchand et al., 2003).

Individual condition is an important component of performance, survivorship and reproductive success in fish (Ferron and Leggett, 1994). Fulton's K condition index has often been used in stress assessment studies because this type of index is based on easily obtained length and weight measurements (Gilliers et al., 2006; Fonsceca et al., 2009). This index is considered a good indicator of the general welfare of the fish and an integrative estimate of environmental conditions (Ferron and Leggett, 1994; Fukuda et al., 2001). The length-weight relationship and Fulton's K condition index of flounder from the Seine estuary were significantly lower than in the other estuaries indicating a lower condition of the fish. Nucleic acid based indices, namely RNA content or the ratio of RNA to DNA content (RNA-DNA), have been used in numerous studies as indices for nutritional condition and growth assessment in juvenile fish (e.g. Buckley, 1999; Fukuda et al., 2001; Gilliers et al., 2006; Humphrey et al. , 2007; Vinagre et al., 2008b; Fonseca et al., 2009). Both RNA concentration and RNA-DNA ratio were lower in the Seine estuary. Lower RNA-DNA values in fish from highly contaminated estuary were previously recorded (Humphrey et al., 2007; Vinagre et al., 2008b; Amara et al., 2009).

Otolith analyses are useful proxy for somatic fish growth. Our data demonstrate that both the otolith peripheral increment widths (recent growth) and the perimeter of the otolith standardised by the fish length showed significant differences among flounders from the Seine estuary compared to the Canche, Authie and Somme estuaries. In fact, results from several studies (Secor and Dean, 1989; Wright et al., 1990; Francis et al., 1993; Hare and Cowen, 1995) suggest that slower-growing fish may have larger otoliths than faster-growing fish of similar sizes or ages. This is not intuitive, but it occurs because slower-growing fish have a higher ratio of mineral to protein in their otoliths, thereby producing heavier, thicker

otoliths (Radtke et al., 1985). The detection of differences between estuarine flounder provides promise for the use of otolith growth indices as tools to screen for habitat-based differences in growth (as suggested by Secor and Dean, 1989).

Fishes' response to environmental and ecological constrains is complex, depending on the intricate relations between environmental conditions and the individual ability to adapt. Nevertheless, the present study emphasizes that the reduced growth and condition of juvenile flounders of the Seine estuary could be the result of the energy cost of detoxification/protection/restoration processes, commonly involved in coping with the chemical stress. The Seine estuary is a good example of a site where human pressures and natural values compete with each other. Although the Seine estuary is highly polluted, this estuary yet continues to support a very high benthic biomass and remains quite productive (Dauvin, 2008; this study). However, surprisingly, this estuary displays low amounts of juveniles fish despite a potential important carrying capacity as nursery area (Riou et al., 2001; Gilliers et al., 2006). The overall impact of poor fish biological performance on fish communities has not been measured, but it is likely that impaired fish health manifest themselves at higher levels of ecological organisation, leading to reduced abundance, production and recruitment of fish in the Seine. The high anthropogenic disturbances may have altered the nursery capacity and functioning and decreased its suitability. Rapid growth may confer ecological advantages and increase survivorship of early juvenile fishes (Sogard, 1992). As a result, variation in the suitability of nursery habitats for juvenile growth can strongly influence successful recruitment to adult populations. As the Seine is the single large estuary of the Eastern Channel, these alterations could affect important fish resources depending on estuarine nursery grounds in this area (LePape et al., 2007).

As a conclusion, the present study emphasized the negative impact of contaminants on the nursery function of estuaries. The combined use of growth and condition indices may provide a useful tool to monitor and assess habitat quality for juvenile fishes, as well as the general ecological status of estuaries. More extensive research is needed in order to evaluate how pollutants are detrimental to flounder populations and more generally to estuarine fishes

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# CHAPTER III - 2

# EFFECTS OF ESTUARY SEDIMENT CONTAMINATION ON PHYSIOLOGY, BIOCHEMICAL BIOMARKERS AND IMMUNE PARAMETERS IN JUVENILE EUROPEAN SEA BASS (*Dicentrarchus labrax*, L., 1758)

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**ABSTRACT**

Estuaries play important ecological function but are generally exposed to anthropogenic impacts. The purpose of this study was to evaluate how estuarine sediment contamination influence juveniles fish using these habitats as nursery grounds. We analysed the responses of both biochemical and physiological biomarkers of juvenile sea bass (*Dicentrarchus labrax*) exposed to fresh sediments from five sites more or less polluted during 21-day laboratory assays. Physiological performance responses (growth rates in length and weight, RNA-DNA ratio, and morphometric (Fulton's K) condition indices), biochemical biomarkers and immune parameters of juveniles from each site were measured at 0, 7 and 21 days. Sediment were analysed for total organic matter, granulometry, metals, PAHs and PCBs contents and fish gill used for metal bioaccumulation measurements. The site of Rouen (in the Seine estuary) showed higher metals and PAH contents in sediment and also higher metal concentrations in gills. They were no significant differences in growth rate in length and weight, Fulton's K

condition index and RNA-DNA ratio among conditions. Among the reference sediment (Wimereux) and the most contaminated sediment (Rouen) any significant differences was observed in spleen and thymus. However, EROD, GST and CAT activities were found significantly higher in Rouen compared to the other conditions. No evident mortality or malformation or signs of toxicity were observed in either condition during the experiment. Our study indicates that the analysis of simultaneous responses of multibiomarkers can be useful for monitoring complex exposure and to assess habitat quality.

Key words: Fish, microcosm study, metals and organic pollutants, bioaccumulation, fish indicators, pollution

III.2.1. Introduction

Coastal transitional zones (e.g. estuaries and coastal lagoons) are amongst the most productive and valuable aquatic ecosystems (Costanza et al., 1997). Out of these zones, estuaries have been characterized as important nursery grounds for many marine fish species (Vasconcelos et al., 2010). Indicated in literature as "essential fish habitat", the capacity and quality of these ecosystems play a key role on juvenile fish growth and survival (Beck et al., 2001). However these habitats are also very sensitive to a wide range of anthropogenic activities such as urban and industrial pollution, agriculture, human settlements, fishing, and port activities that can affect their quality and functioning (Kennish, 2002; Vinaigre et al., 2009; Marchand et al., 2010). Therefore, it is essential to protect these habitats and develop necessary monitoring methods using biochemical indicators to estimate their quality.

The toxic effects of contaminants on aquatic species are often subtle, negatively affecting ecological fitness and consequently reproduction and survival via sublethal cellular, physiological, behavioral or immunological effects. The transfer of contaminants from sediments to biota is obviously a necessary requisite for the occurrence of toxicity. To date, many studies have utilized fish-based in vitro test to assess sediment contamination on exposed marine organisms (e.g. Ensenbach, 1998; Strmac et al., 2002; Hollert et al., 2003). However, controlled laboratory conditions are greatly simplistic compared to the natural environment and substantial extrapolation is required to predict effects for field populations (Chapman et al., 2002; Chapman, 2007). Few studies have integrated indicators of exposure to contamination and effects on fish's health and condition, and most have reported unclear or limited responses of individual growth and condition to contaminant exposure (De Boeck et al., 1997; Wu et al., 2003; Humphrey et al., 2007). In this context, many integrated and multidisciplinary approaches combining chemical, ecotoxicological, and ecological information have been developed and improved to address questions relating to the presence of chemical pollutants, their bioavailability, and their adverse effects and/or potential hazards at different levels of biological organization (Hallare, 2005; Piva, 2011). Then, studies have outlined the importance of applying a multibiomarker approach to assess the causes and effects of stressors on marine systems (e.g. Adams, 2005; Broeg and Lehtonen, 2006).

Biochemical biomarker responses have been applied intensively as early warning signals of contaminant exposure, mainly because of their general acceptance amongst the earliest detectable toxicant-induced responses in aquatic organisms (Peakall, 1992) and have been used to assess habitat quality (Fonseca, 2011). Nevertheless, the presence of complex

mixtures of xenobiotics in the environment and of other potentially confounding factors (i.e. life stage, abiotic natural variability) may result in further difficulties in the interpretation of biomarkers response patterns (Adams, 2002; van Der Oost et al., 2003). A number of studies describe effects on the fish immune system after exposure to environmental pollutants (Robohm, 1986; Secombes et al., 1991; Pulsford et al., 1992; Zelikoff, 1993; Arkoosh et al., 1994; Sanchez-Dardon et al., 1999; Aaltonen et al., 2000), which alters the capability of the individual to cope with pathogen challenges. Furthermore, chemical analyses and/or utilisation of biochemical and immunological analyses alone do not reveal the impact of chemical pollution on the aquatic environment because of potential synergistic/antagonistic effects of complex mixtures of chemical pollutants. The use of physiological biomarkers, such as growth and condition indices are also complementary tools to assess sediment contamination and so habitat quality of juvenile fish. Hence, several measures of growth and condition of larvae and juvenile fish have been used to assess individual and population status as well as habitat quality (e.g. Yamashita et al., 2003; Gilliers et al., 2004; Fonseca et al., 2006; Amara et al., 2009). These measures comprise growth indices (RNA:DNA ratios, protein specific growth rate, otolith growth), morphometric indices (Fulton's K) and storage indices (lipid content) that relate to the individual ability to respond and interact with the environment at different time scales (Suthers, 1998).

In the present study, the assessment of the relationships between chemical contamination, bioavailability and alterations in several biochemical and immunological indicators have been analysed on sea bass juveniles *Dicentrarchus labrax* exposed to different sediment contamination gradient. Sea bass was chosen as an indicator species because it is a common and commercial species with a wide range of distribution along the Mediterranean and European Atlantic coasts. This demersale species lives in shallow coastal areas likely to be impacted by nearshore activities and have a rapid growth particularly during their juvenile stage and change in their physiology due to external stressor easily detectable. Sea bass, living in the water column and feeding on the bottom and the water column, is considered a sensitive fish and it has been shown to tolerate estuarine conditions and a wide range of sediment grain size types. Hatchery production of sea bass has lead to an increased availability of individuals with a known exposure history, which is an essential element in any toxicological study (Boisson et al., 1998).

Juvenile sea bass were exposed to five different gradient sediment contaminations for 21 day representing both polluted areas and a relatively unpolluted reference location.

Environmental conditions such as hydrological parameters, metals, PAHs, PCBs, organic matter contents and granulometry in sediment were analysed and metal bioaccumulation in gills were also measured on juvenile European sea bass compared among the five sites. The growth rate and condition indices (Fulton's K condition index, RNA:DNA ratio) and the relationship with other biomarkers and immune responses were explored in different sediment contamination level.

### III.2.2. Materials and Methods

The experiment was conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. The University of Littoral Côte d'Opale is authorized to conduct experimentation on animals in its capacity as a certified establishment; according to the administrative order N° B62-160-2.

#### III.2.2.1. Sediment collection

Five sites were selected along the French coasts of the Eastern English Channel for sediment collection: Site 1 (Wimereux), defined as reference site and less contaminated with relevant pollutants; Site 2 (Canche estuary) a low impacted estuary and three sites located along the Seine estuary, Site 3 (Normandie Bridge), Site 4 (Caudebec) and Site 5 (Rouen) as increasingly polluted sites (Figure 28). Previous data have demonstrated Rouen to be the most contaminated site with high levels of metals, organic compounds compared to Normandie Bridge and Caudebec (Munschy et al., 1997; Carpentier et al., 2002; Meybeck et al., 2004; Cachot et al., 2006). The Canche estuary is not impacted by any important human activity and is considered as clean estuary (Durou et al., 2007; Amara et al., 2007). Sediment on each site was collected with a plastic spatula from surface up to a depth of ~10 cm during low tide and stored in polyethylene bags. About 10 L of sediment of each condition was transported to the laboratory. The fractions samples dedicated to chemical analysis was frozen and stored at – 20 °C. The remainder of the sediment was stored at -4°C during few days until the exposure experiment.

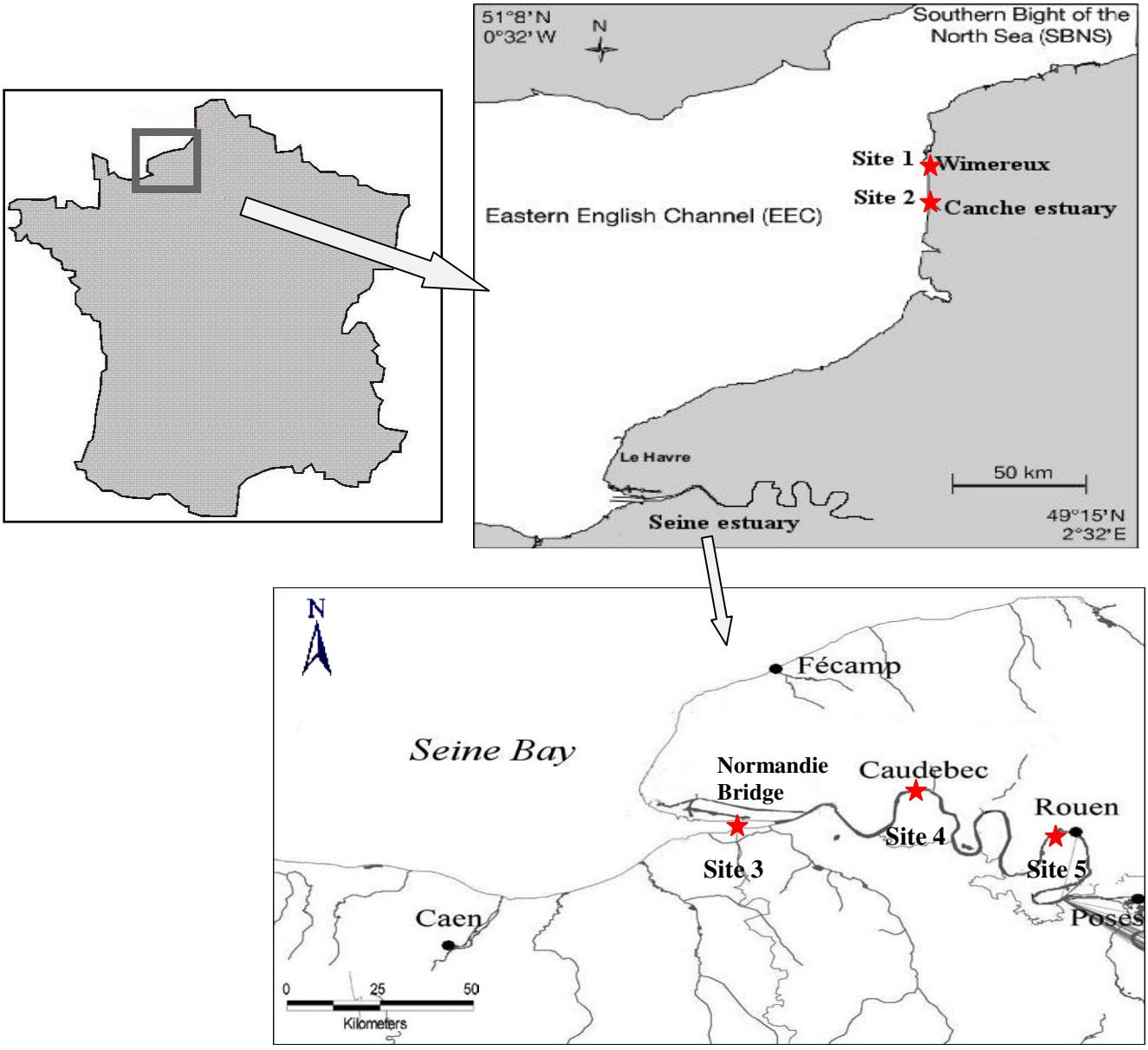



Figure 28. Locations of the five collecting sediment sites (★ Wimereux, Canche, Normandie Bridge, Caudebec and Rouen)

III.2.2.2. Fish and experimental set up

Juvenile European sea bass (*Dicentrarchus labrax*), 100 days old, were obtained from Aquanord hatchery (Graveline, France). Fish, with a mean initial total length 54.5 ± 3.8 mm and weight 1423.6 ± 365.8 mg were acclimatised for two weeks in two 160 L clean tanks (n=115 fish/tank). Following the acclimatization, sea bass juveniles were anaesthetised (2-

phenoxyethanol), weighed (near to 10 mg), measured for total length (near to 0.1 mm), and individually marked (Visual Implant Tag, 1.2 mm x 2.7 mm, Northwest Marine Technology, USA).

Two of ten 30 L aquariums were filled with 5 L sediment from one site and filled up with natural filtered sea water. Sediment let settled during 2 days to avoid release of suspended particles in the water. 20 fish were randomly distributed into each aquarium. The experiment was conducted in semi-static conditions. One-third of the seawater in each tank was renewed manually every day. Each tank was aerated continuously and water temperature in tanks was kept constant at 14 ± 1°C. The photoperiod was set at 10 h light and 14 h dark cycle. Fish were fed 1% of the mean body weight once a day with commercial dry pellets. Oxygen (mg/l), temperature (°C), salinity, pH (Hanna HI 9828 multiprobe) and turbidity (NTU) (Eutech instruments, TN-100) measurements were recorded every day before feeding and renewing seawater.

Thirteen fish were sampled at the beginning of the experiment as reference ($t_0$). For biomarker analyses after one week of exposure ($t_7$) livers of 10 fish per aquarium (20 per condition) were sampled, frozen in nitrogen liquid and stored at -80°C. In order to monitor growth and physiological performances of juveniles, 10 individuals per aquarium were removed at the end of experiment ($t_{21}$), rapidly anaesthetised, identified (tag), weighed, and measured for total length. Muscles and gills were stored at −20 °C, respectively, for biochemical and metal bioaccumulation analysis.

### III.2.2.3. Sediment analysis

Sediment samples of each treatment were analysed to determine granulometry, organic matter, metals, PAHs and PCBs before the distribution into the aquariums.

The grain size distribution of the upper 15 cm layer was analysed in humid aliquot of each sediment sample using a laser Beckman-Coulter LS 230. A classification was established according to proportion of clay (<4 µm), fine silt (4-20 µm), coarse silt (20-50 µm), fine sand (50-200 µm), medium sand (200 – 500 µm) and coarse sand (500 – 2000 µm). To measure total organic matter (TOM, $mg.g^{-1}$), sediment samples (~ 2 g) were dried at 60°C for 72 h and subsequently burned at 500°C for 6h. In addition, the total organic carbon (TOC) and total organic nitrogen (TON) contents were determined using a CHNS analyzer (NA 2100, CE instruments).

In order to quantify selected metals (Al, Cd, Cr, Cu, Mn, Ni, Pb, V and Zn) in the total and potentially reactive towards biota fractions, sediments were dried at 40°C in an oven to constant weight and were ground into a powder. For the determination of total metals, about 0.250 g ground sediments were digested with HF (Suprapur, Merck) at 110 °C for 48 h followed by a mixture of concentrated acids HCl:$HNO_3$ (3:1 v:v, Suprapur Merck) at 120°C for 24 h, operation renewed once. Metals associated with the reactive fractions of sediment, considered as bioavailable metals, were obtained using the method of Huerta-Diaz and Morse (1990). The reactive fraction comprises metal exchangeable and the linked to carbonates and partially to oxy-hydroxides of Fe-Mn and to acid volatile sulfides (AVS). About 0.5 g of sediment was leached during 24 h with 20 mL of 1 M HCl (Suprapur, Merck). The total and extractable metals were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES, VARIAN Vista Pro, axial view). For quality assurance, reagent blanks, sample replicates and standard references (MESS-3 and PACS-2, National Research Council Canada) were used to assess the accuracy and precision of the analysis. In all cases, the recovery efficiency was higher than 85 % for the total digestion of these standards.

The persistent organic pollutants, including PAHs (EPA's 16 priority PAHs) and PCBs (7 congeners) were analysed. Organic compounds were extracted from 2 g dried sediment by microwave (120 °C for 15 min, 1200 W) assisted extraction with 40 mL mixture of acetone:hexane (1:1, v:v). The solvent was evaporated under a stream of nitrogen in a TurboVap and then concentrated to 1 mL of hexane. Simultaneous determination of PAHs and PCBs was performed on a gas chromatography–mass spectrometry (GC–MS, VARIAN, CP 3800 – 1200 MS TQ). A ZB–MultiResidue column (30 m, 0.25 mm, 0.25 µm) were used (Phenomenex). Identification of PAH compounds and PCB congeners was based on the comparison of their GC–retention times and their mass spectrum, with appropriate individual standards.

### III.2.2.4. Physiological parameters

In order to distinguish short term experimentally sublethal effects of contaminated sediment on the physiological performances of sea bass juveniles, morphometric and RNA-DNA ratio measurements were determined individually on a total of 113 juveniles for $t_0$ and $t_{21}$ (13 fish for $t_0$ and 10 fish / aquarium for $t_{21}$).

Juveniles sea bass growth rates in weight (mg per day) were estimated as: $GW = (W_2 - W_1)/(t_2 - t_1)$, where ($W_1$) and ($W_2$) are fish total body weight at times $t_1$ (beginning of the experiment) and $t_2$ (time of collection). Similarly, the specific growth rate in length was estimated as: $GL = (L_2 - L_1)/(t_2 - t_1)$, where ($L_1$) and ($L_2$) are fish total length at times ($t_1$) and ($t_2$) respectively.

We estimated two condition indices in fish exposed to contaminated sediment and in fish sacrificed at the beginning of the experiment ($t_0$): RNA:DNA ratio as indicators of nutritional status and Fulton's K condition index as an indicator of the fishes general well being. We calculated Fulton's K condition index with the formula:
$K = 100(W/L^3)$, where (W) is the body mass (mg) and (L) is the total length (mm).

RNA-DNA concentrations in individual fish was based on Clemmesen method (1988), slightly modified as described by Amara et al. (2009). RNA-DNA quantification was accessed by fluorescence-photometric technique using a specific nucleic acid fluorescent dye−ethidium bromide.

### III.2.2.5. Molecular biomarker analysis

Three biomarkers were selected: (e.g. detoxification enzymes: Ethoxyresorufin-O-deethylase (EROD), Glutathione S-transferase, (GST) and oxidative stress enzymes: catalase (CAT)). These biomarkers were analysed in liver of fish collected at $t_7$.

Livers were homogenised in an ice-cold phosphate buffer (0.1 M, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were centrifuged at 10.000 g at 4 °C, for 15 min and the post-mitochondrial fractions were used for biochemical assays. Total protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard.

EROD activity was determined following the hydroxylation of 7-ethoxyresorufin by the method of Flammarion et al. (1998). The reaction mixture consisted of a phosphate buffer (0.1 M, pH 6.5), 7-ethoxyresorufin (8 µM) and NADPH (0.5 mM). The change in fluorescence was recorded (excitation wavelength 530 nm, emission wavelength 585 nm) and enzyme activity calculated as $pmol.min^{-1}.mg^{-1}$ protein using a resorufin standard.

GST activity (GST; EC 2.5.1.18) was determined following the conjugation of reduced glutathione with CDNB by the method of Habig et al. (1974). The reaction mixture

consisted of phosphate buffer (0.1 M, pH 6.5), reduced glutathione (1 mM) and CDNB (1 mM). The change in absorbance was recorded at 340 nm and enzyme activity calculated as $\mu mol.min^{-1}.mg^{-1}$ protein using GST standard.

CAT activity (CAT; EC 1.11.1.6) activity was determined by the method of Babo and Vasseur (1992). In brief, the assay mixture consisted of phosphate buffer (100 mM pH 6.5) and H2O2 (28 mM). Change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of $\mu mol.min^{-1}.mg^{-1}$ protein, using bovine erythrocyte catalase as standard.

### III.2.2.6. Metal analysis in gills

Because of the constraint of gill size, ten fish gills were pooled so two samples of gill were analysed for each condition on $t_0$ and $t_{21}$. The gills were rinsed with Milli-Q water, mixed and lyophilised for analysis of metal concentrations. Approximately 0.08 g of aliquots was digested with ~ 1ml nitric acid (65%, Suprapur Merck). Each metal on gill samples was determined by inductively coupled plasma-mass spectrometry (ICP-MS; VARIAN 820). Standard curves were used to determine Mn and Zn in diluted samples, whereas standard addition technique was applied for resolution of matrix effects to calculate Cd, Cr, Cu, Mn, Ni, Pb, V, Zn, As and Se. International certified standard (DORM–3, NRC Canada) was used to control the accuracy of the analytical procedure.

### III.2.2.7. Histology

Histology was only applied on fish sacrificed at the end of the experiment. A comparison was carried out between the highest polluted site (Rouen) and the control fish (Wimereux). Due to the tiny thymus size, the head section containing the thymic tissue was sampled entirely, while the spleen was dissected apart. The tisssus were either directly fixed in buffered 4% Formaldehyde – Solution (spleen) or stored in Ethanol (100%) for 7 days (thymus) before further dissection and fixation in buffered 4% Formaldehyde – Solution. The head section including the thymus was decalcified in 22% formic acid solution for 5 days. The tissues were dehydrated automatically in a tissue processor (Peloris, Medite, Nunningen, Switzerland) and embedded in paraffin. 5µm thick sections were cut with a microtome (Leica) and transferred to poly-L-lysine (Sigma, France) coated glass slides. Machine-supported

(COT20, Medite) HES (Haematoxylin-Eosin-Safran) stained cuts were observed with Leica QWin Version 2.5.

The number of melanomacrophage centers per area in spleen (10 random cuts per 5 fish of each treatment) was investigated. In thymus, the volumes of the whole organ, cortex and medulla ($mm^3$) and the ratio cortex /medulla were determined. Both thymi per fish (n=5) were examined and a section every $100^{th}$ µm was studied (Casteleyn et al., 2007).

#### III.2.2.8. Statistical analysis

Statistics were performed with Xlstat 2007. Since biological and histological data considered here did not comply with the parametric assumption of normality and variance equality, non-parametric Kruskall–Wallis test followed by the post hoc Dunn test (joint ranking test) were used for pair wise comparisons. Mean comparisons of molecular biomarker responses between $t_0$ and other conditions were analysed using one-way ANOVA, followed by post hoc Tukey tests. Normality and homoscedasticity were tested using, respectively, Kolmogorov-Smirnov test (using Shapiro-Wilk and Lilliefors corrected tables). A principal component analysis (PCA) was used to evaluate the relationships between the chemical sediment contaminants, biomarkers and fish biological responses.

### III. 2. 3. Results

#### III.2.3.1. Environmental parameters

All measured physico-chemical parameters were kept stable over the 21 days exposure, unless turbidity (Table 7). There are evident variations for turbidity with the highest discrepancy for Normandie Bridge and Rouen. Sediment grain size composition was 99% mainly formed by sand (fine, medium, coarse). On the contrary, mud (clay, fine and coarse silt) was the main component of sediment from Canche (62%), Caudebec (70%), Normandie Bridge (81%) and Rouen (83%). The load for organic contents (TOM, TOC and TON) was lower in reference sediment compared to other sites.

Sediment samples indicated different levels of contamination in metallic and organic compounds. The measured values for metals, PAHs and PCBs expressed as mg. $kg^{-1}$ of dry sediment are reported for each site in Table 8. As expected, Wimereux sediment exhibited the

lowest metal concentrations and PAHs concentrations were below detection limits. Canche and Caudebec sites showed close metal contamination for some elements (Cd, Cr, Cu, Ni and V). Rouen defined as the most polluted site is loaded with the highest metal concentrations. PAHs concentration in Canche site was 0.12 ± 0.01 $mg.kg^{-1}$. The Seine estuary sites have different concentrations of PAHs with higher values in Rouen (5.91 ± 0.20 $mg.kg^{-1}$) compared to Normandie Bridge (1.63 ± 0.05 $mg.kg^{-1}$) and Caudebec (1.30 ± 0.04 $mg.kg^{-1}$). In all sites, no PCBs could be detected because of the sensibility limit of the method employed (0.01 $mg.kg^{-1}$ to determine one congener content). Difference of bioavailability among elements was detected among estuary sites: Cd, Cu, Mn, Pb and Zn showed a relatively strong affinity with the acid-soluble fraction, which represents more than 50 % while Cr, Ni and V remained lower than 30 %. The proportion of bioavailability of metals was higher in Rouen (except for Cr and V) compared to the other sites (Figure 29).

The numerical, effects-based, of contamination sediment quality guidelines were performed to determine the sediment toxicity. Long et al. (1995) identified two guidelines values: the effects range-low (ERL) and the effects range-median (ERM), described three ranges in chemical concentrations for 25 compounds where adverse effects were rarely (< ERL); 2) occasionally (ERL≤ x < ERM) or frequently observed (x ≥ ERM). For comparison, Table 2 gives also these guideline values for trace metals and organic compounds selected in the present study. Using this classification, the chemical contaminants detected in Wimereux, Canche, Normandie Bridge and Caudebec were lower that the ERL. On the other hand, five metal concentrations (Cd, Cu, Ni, Pb, and Zn) and nine PAHs contents in sediment from Rouen are classified between ERL and ERM. The effects-ranged guidelines and chemical contamination showed that some elements in Rouen exhibited occasional levels of toxicity whereas other sites seem to have good criteria of sediment quality.

Table 7. Mean (±SD) values of abiotic and biotic parameters in the five sites during acclimatisation and exposure period. TOM : total organic matter; TON : total organic nitrogen; TOC : total organic carbon concentrations

| | Acclimatization tanks | | Exposure aquariums | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | Wimereux | Canche | Normandie Bridge | Caudebec | Rouen |
| **Environmental parameters** | | | | | | | |
| Temperature (°C) | 15.0 ± 0.5 | 15.0 ± 0.5 | 14.72 ± 0.28 | 14.62 ± 0.29 | 14.62 ± 0.36 | 14.59 ± 0.31 | 14.60 ± 0.33 |
| Salinity | 34.5 ± 0.6 | 34.6 ± 0.3 | 33.28 ± 0.67 | 33.20 ± 0.70 | 33.46 ± 0.43 | 33.31 ± 0.69 | 33.32 ± 0.74 |
| Oxygen ($mg.l^{-1}$) | 8.3 ± 0.7 | 8.4 ± 0.5 | 8.09 ± 0.77 | 8.34 ± 0.56 | 8.01 ± 0.59 | 8.03 ± 0.57 | 8.11 ± 0.55 |
| pH | 7.8 ± 0.1 | 7.8 ± 0.1 | 7.84 ± 0.09 | 7.88 ± 0.09 | 7.81 ± 0.08 | 7.86 ± 0.10 | 7.87 ± 0.11 |
| Turbidity (NTU) | | | 1.22 ± 0.64 | 57.71 ± 52.72 | 86.80 ± 66.56 | 42.57 ± 45.50 | 80.77 ± 66.36 |
| **Sediment** | | | | | | | |
| TOM ($mg\ g^{-1}$) | | | 3.83 | 32.80 | 55.32 | 24.96 | 133.80 |
| TOC ($mg\ g^{-1}$) | | | 2.29 | 10.9 | 14.52 | 4.2 | 46.42 |
| TON ($mg\ g^{-1}$) | | | 0.03 | 1.32 | 3.9 | 1.01 | 7.19 |
| Clay % | | | 0.2 ± 0.0 | 9.0 ± 1.0 | 15.3 ± 0.5 | 8.1 ± 0.7 | 8.6 ± 0.2 |
| Fine silt % | | | 0.4 ± 0.0 | 23.3 ± 2.7 | 33.4 ± 1.4 | 26.1 ± 0.9 | 43.1 ± 0.7 |
| Coarse silt % | | | 0.6 ± 0.1 | 29.6 ± 0.9 | 31.9 ± 0.7 | 36.0 ± 2.5 | 31.1 ± 0.8 |
| Fine sand% | | | 56.9 ± 0.4 | 38.1 ± 4.6 | 19.4 ± 2.4 | 29.7 ± 2.3 | 17.2 ± 1.5 |
| Medium sand % | | | 38.4 ± 0.4 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.0 ± 0.0 |
| Coarse sand % | | | 3.6 ± 0.2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

Table 8. Mean (± SD) sediment metal, PAHs and PCBs concentrations in the five sites and sediment quality guideline values (in mg $kg^{-1}$ dry weight)

| Substance | | Concentration in sediment | | | | | Guidelines | |
|---|---|---|---|---|---|---|---|---|
| | | Wimereux | Canche | Normandie Bridge | Caudebec | Rouen | ERL | ERM |
| Metals | | | | | | | | |
| | Cd | 0.13 ± 0.01 | 0.32 ± 0.01 | 0.65 ± 0.02 | 0.54 ± 0.05 | 1.59 ± 0.01 | 1.2 | 9.6 |
| | Cr | 8.58 ± 0.13 | 29.7 ± 0.5 | 48.2 ± 0.7 | 33.7 ± 2.9 | 74.5 ± 1.1 | 81 | 370 |
| | Cu | 0.98 ± 0.01 | 7.31 ± 0.04 | 16.3 ± 0.2 | 11.5 ± 0.2 | 65.4 ± 0.5 | 34 | 270 |
| | Mn | 74.2 ± 0.4 | 237 ± 3 | 325 ± 1 | 302 ± 4 | 533 ± 3 | - | - |
| | Ni | 1.65 ± 0.01 | 7.89 ± 0.31 | 11.9 ± 0.3 | 8.40 ± 0.46 | 28.0 ± 0.1 | 20.9 | 51.6 |
| | Pb | 5.23 ± 0.19 | 12.5 ± 1.8 | 26.9 ± 0.4 | 20.6 ± 1.3 | 68.8 ± 2.6 | 46.7 | 218 |
| | V | 7.40 ± 0.01 | 24.57 ± 0.06 | 38.16 ± 0.21 | 23.28 ± 0.46 | 70.17 ± 0.02 | - | - |
| | Zn | 9.67 ± 0.07 | 43.9 ± 1.2 | 79.4 ± 0.8 | 65.8 ± 1.7 | 281 ± 2 | 150 | 410 |
| | Al | 10179 ± 152 | 19491 ± 10 | 22879 ± 2745 | 17783 ± 4203 | 42509 ± 601 | - | - |
| PAHs | | | | | | | | |
| | Naphthalene | < 0.05 | < 0.05 | 0.07 ± 0.01 | < 0.05 | 0.10 ± 0.01 | 0.16 | 2.10 |
| | Acenaphtylene | < 0.05 | < 0.05 | < 0.05 | < 0.05 | < 0.05 | 0.04 | 0.64 |
| | Acenapthene | < 0.05 | < 0.05 | < 0.05 | < 0.05 | < 0.05 | 0.02 | 0.50 |
| | Fluorene | < 0.05 | < 0.05 | < 0.05 | < 0.05 | 0.08 ± 0.01 | 0.02 | 0.54 |
| | Phenanthrene | < 0.05 | < 0.05 | 0.12 ± 0.01 | 0.09 ± 0.01 | 0.37 ± 0.02 | 0.24 | 1.50 |
| | Anthracene | < 0.05 | < 0.05 | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.21 ± 0.02 | 0.09 | 1.10 |
| | Fluoranthene | < 0.05 | 0.06 ± 0.01 | 0.20 ± 0.01 | 0.16 ± 0.01 | 0.85 ± 0.02 | 0.60 | 5.10 |
| | Pyrene | < 0.05 | < 0.05 | 0.17 ± 0.01 | 0.14 ± 0.01 | 0.68 ± 0.01 | 0.67 | 2.60 |
| | Benzo [a] anthracene | < 0.05 | < 0.05 | 0.14 ± 0.01 | 0.14 ± 0.01 | 0.51 ± 0.04 | 0.26 | 1.60 |
| | Chrysene | < 0.05 | < 0.05 | 0.16 ± 0.01 | 0.13 ± 0.01 | 0.49 ± 0.03 | 0.38 | 2.80 |
| | Benzo [b] fluoranthene | < 0.05 | 0.06 ± 0.01 | 0.22 ± 0.01 | 0.16 ± 0.01 | 0.85 ± 0.01 | - | - |
| | Benzo [k] fluoranthene | < 0.05 | < 0.05 | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.31 ± 0.05 | - | - |
| | Benzo [a] pyrene | < 0.05 | < 0.05 | 0.09 ± 0.01 | 0.11 ± 0.01 | 0.53 ± 0.01 | 0.43 | 1.60 |
| | Indeno [1,2,3-cd] pyrene | < 0.05 | < 0.05 | 0.15 ± 0.02 | 0.11 ± 0.02 | 0.44 ± 0.07 | - | - |
| | Benzo [ghi] perylene | < 0.05 | < 0.05 | 0.10 ± 0.01 | 0.08 ± 0.01 | 0.41 ± 0.01 | - | - |
| | Dibenzo [a,h] antrachene | < 0.05 | < 0.05 | 0.07 ± 0.05 | 0.06 ± 0.04 | 0.13 ± 0.09 | 0.06 | 0.26 |
| | Total PAHs | | 0.12 ± 0.01 | 1.63 ± 0.05 | 1.30 ± 0.04 | 5.91 ± 0.20 | - | - |
| PCBs | | | | | | | | |
| | Total PCBs (congeners 28 – 52 – 101 – 118 – 138 – 153 – 180) | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | 0.02 | 0.18 |

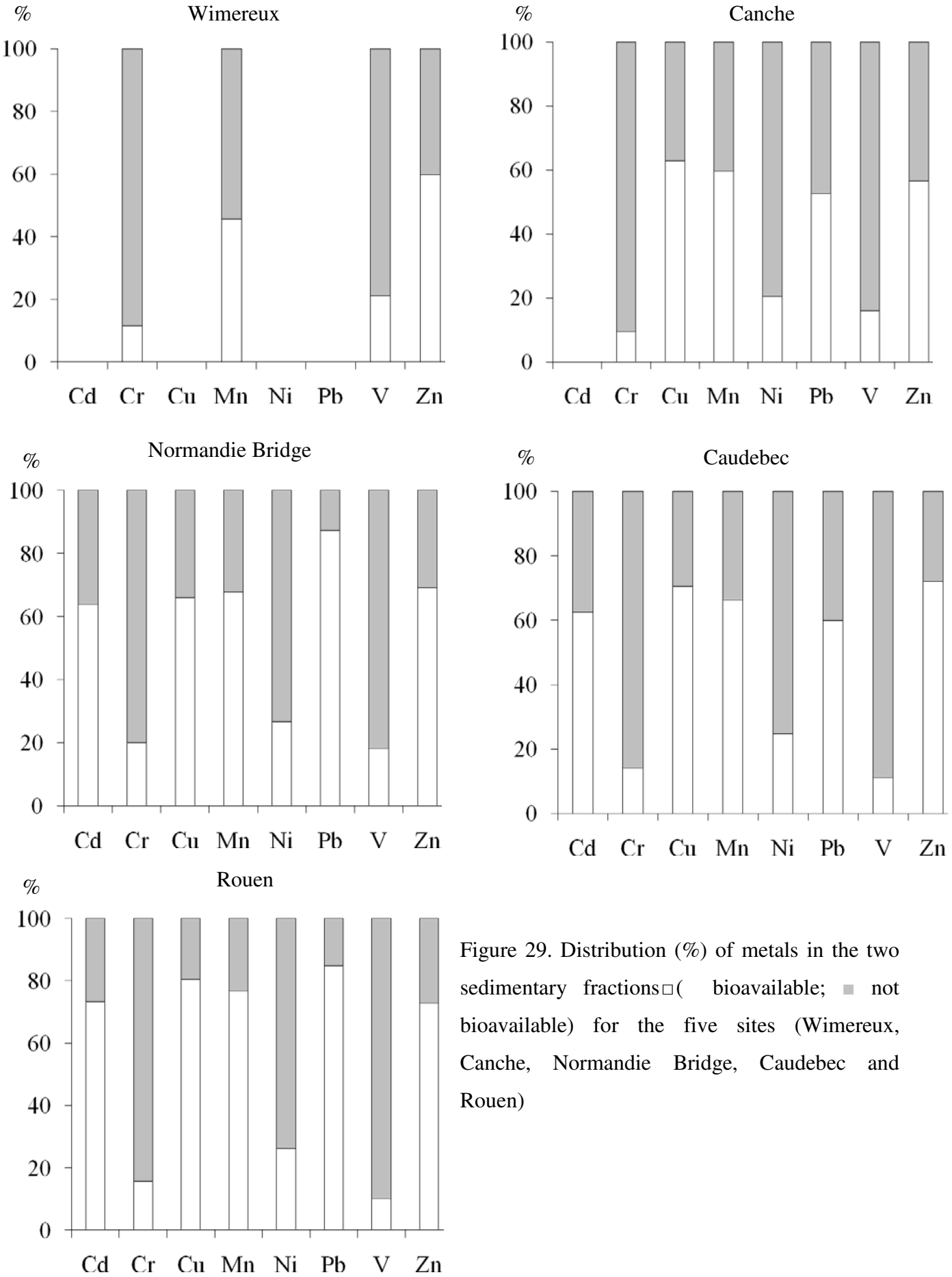



Figure 29. Distribution (%) of metals in the two sedimentary fractions (□ bioavailable; ■ not bioavailable) for the five sites (Wimereux, Canche, Normandie Bridge, Caudebec and Rouen)

### III.2.3.2. Physiological parameters

It was observed dead individual considering six fish during the acclimatization, eleven fish during the tagging procedure and one in Caudebec and three in Rouen aquariums during exposure.

Growth of juvenile sea bass was similar between both replicates and there was no significant difference in fish size and weight among conditions over the experiment (KW, $p>0.05$) (Table 9). During the 21 days of microcosm experiment, the size of fish juveniles increased from 53.7 ± 4.1 mm (TL) to 59.2 ± 4.8 mm and the weight from 1363.2 ± 348.6 to 2132.2 ± 557.0 mg. Growth rates in length and weight stayed constant among the different exposure scenario and fluctuate from 0.15 to 0.19 (mm $d^{-1}$) and from 27.54 to 31.57 (mg $d^{-1}$) respectively.

The Fulton's K condition index varied between 0.86 ± 0.09 and 1.04 ± 0.08 mg $mm^{-3}$. After three week ($t_{21}$), K values were significantly higher in the five sites compared to $t_0$ (KW, $p<0.0001$) and no significant differences of K values were recorded between conditions (KW, $p>0.05$).

The RNA-DNA ratio varied between 2.00 ± 1.65 and 2.71 ± 0.59 and showed no significant differences between conditions (KW, $p=0.308$). Individuals from Normandie Bridge, Caudebec and Rouen exhibited higher RNA-DNA ratio while those of the Canche indicated lower values compared to Wimereux.

### III.2.3.3. Metal concentrations in gills

After 21 days of exposure with different sediment pollution gradients, concentrations of trace metals in gills are reported in Table 10 for $t_0$ and $t_{21}$ on sea bass juveniles. Due to few gill material (two replicates of a mixture of ten gills per station), no statistical analysis could be done to compare metals analysed between conditions. Metal concentrations for $t_0$ showed similar values compared to those for Wimereux exposition (except Ni, Pb and Zn). Metal accumulation in sea bass gill of Rouen exhibited higher concentrations for Cr, Cu, Pb, V and Se compared to other exposure sediment sites.

Table 9. Mean (± SD) values of biological parameters measured on sea bass juveniles in the five sites. Depicted parameters are total length (TL, mm), growth rate in length (GL; mm $d^{-1}$), body weight (W, mg), growth rate in weight (GW, mg $d^{-1}$), Fulton's condition index (K), RNA-DNA ratio. Superscript ($^1$) stands for significant difference compared to "$t_0$"

| Days | Conditions | Total length (TL, mm) | GL (mm $d^{-1}$) | Body weight (W, mg) | GW (mg $d^{-1}$) | K (mg $mm^{-3}$) | RNA-DNA ratio |
|---|---|---|---|---|---|---|---|
| $t_0$ | - | 53.7 ± 4.1 | - | 1363.2 ± 348.6 | - | 0.86 ± 0.09 | 2.66 ± 0.89 |
| | Wimereux | 58.7 ± 3.8 | 0.19 ± 0.08 | 2074.3 ± 462.1 | 31.57 ± 10.66 | 1.01 ± 0.06$^1$ | 2.20 ± 1.39 |
| | Canche | 57.3 ± 4.8 | 0.16 ± 0.07 | 1991.7 ± 505.3 | 27.54 ± 8.07 | 1.04 ± 0.05$^1$ | 2.00 ± 1.65 |
| $t_{21}$ | Normandie Bridge | 59.2 ± 4.8 | 0.16 ± 0.08 | 2132.2 ± 557.0 | 30.31 ± 11.07 | 1.01 ± 0.06$^1$ | 2.42 ± 0.98 |
| | Caudebec | 57.5 ± 4.3 | 0.15 ± 0.14 | 2013.8 ± 497.8 | 30.26 ± 10.19 | 1.04 ± 0.08$^1$ | 2.70 ± 1.00 |
| | Rouen | 58.0 ± 4.6 | 0.16 ± 0.06 | 2069.6 ± 555.5 | 27.92 ± 10.86 | 1.04 ± 0.06$^1$ | 2.71 ± 0.59 |

Table 10. Mean (± SD) metal concentrations (mg $kg^{-1}$) in gills of sea bass juveniles in the five sites

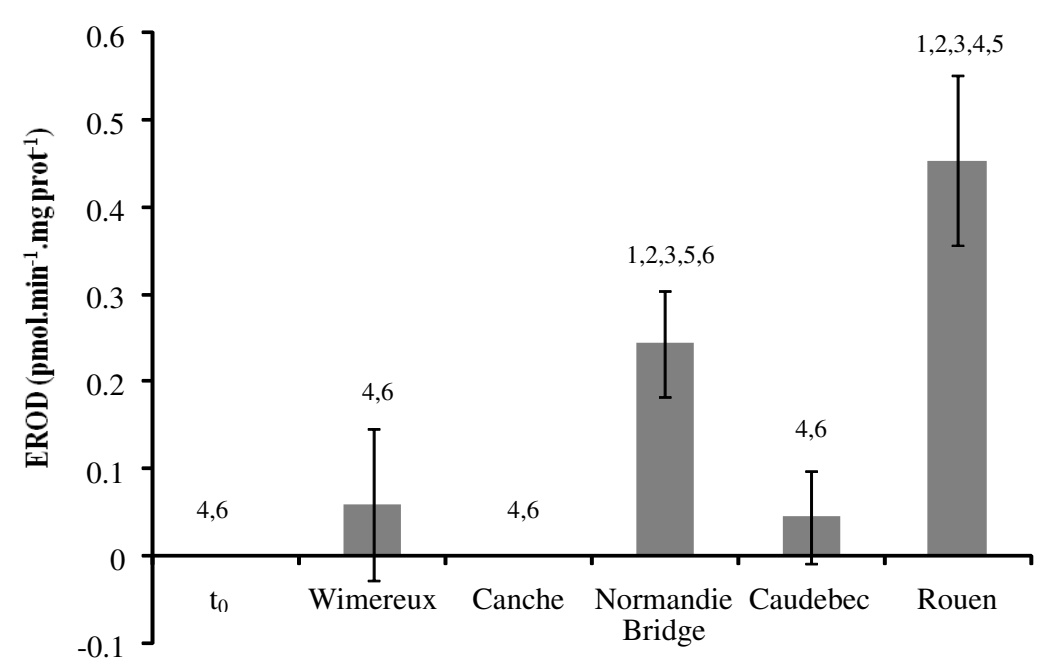
| | Metals | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sites | Cd | Cr | Cu | Mn | Ni | Pb | V | Zn | As | Se |
| $t_0$ | 0.03 ± 0.01 | 0.95 ± 0.15 | 3.76 ± 0.35 | 16.12 ± 1.34 | 0.42 ± 0.09 | 0.23 ± 0.11 | 0.56 ± 0.01 | 142 ± 1 | 2.57 ± 0.01 | 3.69 ± 0.20 |
| $t_{21}$ | | | | | | | | | | |
| Wimereux | 0.03 ± 0.01 | 0.83 ± 0.06 | 3.49 ± 0.03 | 14.26 ± 0.99 | 0.26 ± 0.02 | 0.16 ± 0.01 | 0.58 ± 0.01 | 133 ± 2 | 2.61 ± 0.11 | 3.22 ± 0.48 |
| Canche | 0.03 ± 0.01 | 1.09 ± 0.06 | 3.29 ± 0.68 | 16.49 ± 2.21 | 0.27 ± 0.01 | 0.23 ± 0.04 | 0.69 ± 0.01 | 125 ± 9 | 2.68 ± 0.21 | 3.90 ± 0.45 |
| Normandie Bridge | 0.02 ± 0.01 | 0.96 ± 0.29 | 3.33 ± 1.76 | 18.49 ± 2.17 | 0.28 ± 0.04 | 0.32 ± 0.16 | 0.63 ± 0.17 | 127 ± 4 | 2.25 ± 0.83 | 3.35 ± 1.49 |
| Caudebec | 0.04 ± 0.01 | 1.04 ± 0.06 | 3.24 ± 0.63 | 17.81 ± 0.02 | 0.25 ± 0.05 | 0.27 ± 0.05 | 0.62 ± 0.08 | 125 ± 8 | 2.98 ± 0.24 | 4.27 ± 0.62 |
| Rouen | 0.03 ± 0.01 | 1.16 ± 0.01 | 4.46 ± 0.17 | 17.77 ± 3.19 | 0.31 ± 0.01 | 0.55 ± 0.16 | 0.83 ± 0.11 | 123 ± 2 | 2.86 ± 0.03 | 4.46 ± 0.08 |

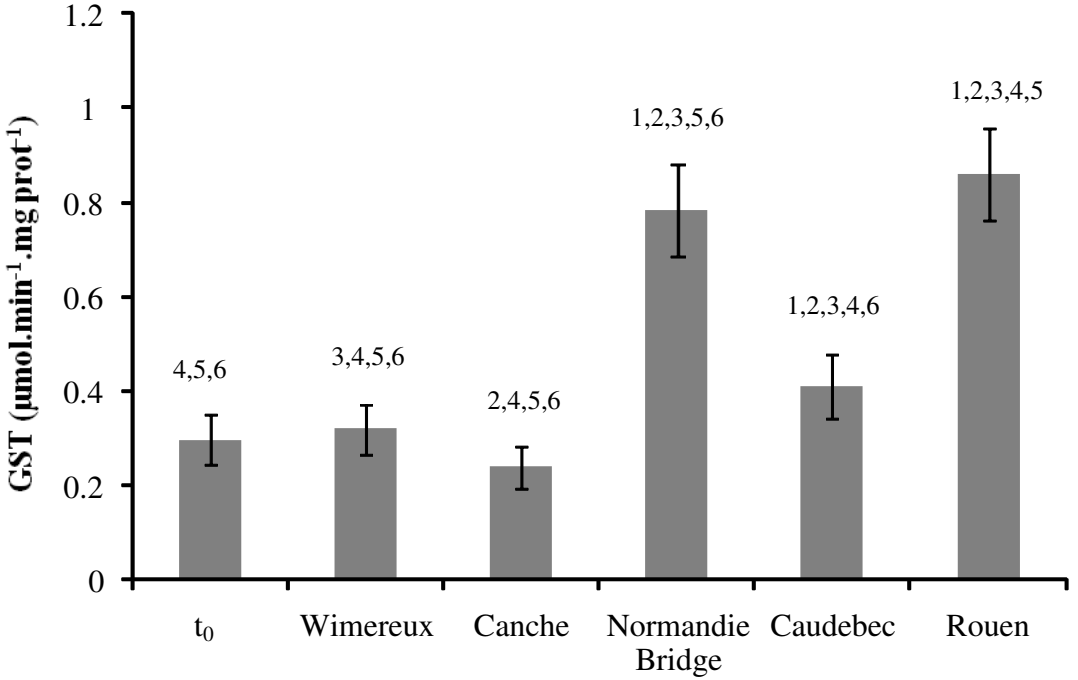
III.2.3.4. Biomarker responses

Three hepatic biomarkers measured in sea bass juveniles are shown in Figure 30. EROD activity measured in sea bass sacrificed at the beginning of the experiment ($t_0$) and those in Canche were below the detection limit, whereas GST and CAT activities were detected in all sites. Values of EROD, GST and CAT activities induced proportionally to the contaminated site, Rouen, compared to other conditions. A significant induction of EROD activity was observed in fish from Rouen (0.45 ± 0.10 pmol $min^{-1}$ $mg^{-1}$ protein) compared to Normandie Bridge (0.24 ± 0.06 pmol $min^{-1}$ $mg^{-1}$ protein), Wimereux (0.06 ± 0.09 pmol $min^{-1}$ $mg^{-1}$ protein), Caudebec (0.05 ± 0.05 pmol $min^{-1}$ $mg^{-1}$ protein), Canche (0.00 ± 0.00 pmol $min^{-1}$ $mg^{-1}$ protein) and $t_0$ (0.00 ± 0.00 pmol $min^{-1}$ $mg^{-1}$ protein), respectively (Anova, $p<0.0001$).

Mean GST activity showed significantly higher values in Rouen (0.86 ± 0.10 µmol.$min^{-1}$ $mg^{-1}$ protein) compared to Normandie Bridge (0.78 ± 0.10 µmol $min^{-1}$ $mg^{-1}$ protein), Caudebec (0.41 ± 0.07 µmol $min^{-1}$ $mg^{-1}$ protein), Wimereux (0.32 ± 0.05 µmol $min^{-1}$ $mg^{-1}$ protein), $t_0$ (0.30 ± 0.05 µmol $min^{-1}$ $mg^{-1}$ protein) and Canche (0.24 ± 0.04 µmol $min^{-1}$ $mg^{-1}$ protein), respectively (Anova, $p<0.0001$). On the contrary, a significant decrease of GST activity was measured in Canche (Anova, $p=0.008$) compared to Wimereux and the three Seine estuary sites (Anova, $p<0.0001$).

Catalase activities were significantly higher in Rouen (1.32 ± 0.14 µmol $min^{-1}$ $mg^{-1}$ protein) compared to Caudebec (1.20 ± 0.10 µmol $min^{-1}$ $mg^{-1}$ protein), Normandie Bridge (1.11 ± 0.16 µmol $min^{-1}$ $mg^{-1}$ protein), $t_0$ (1.01 ± 0.09 µmol $min^{-1}$ $mg^{-1}$ protein), Wimereux (1.00 ± 0.10 µmol $min^{-1}$ $mg^{-1}$ protein) and Canche (0.87 ± 0.11 µmol $min^{-1}$ $mg^{-1}$ protein), respectively (Anova, $p<0.0001$).



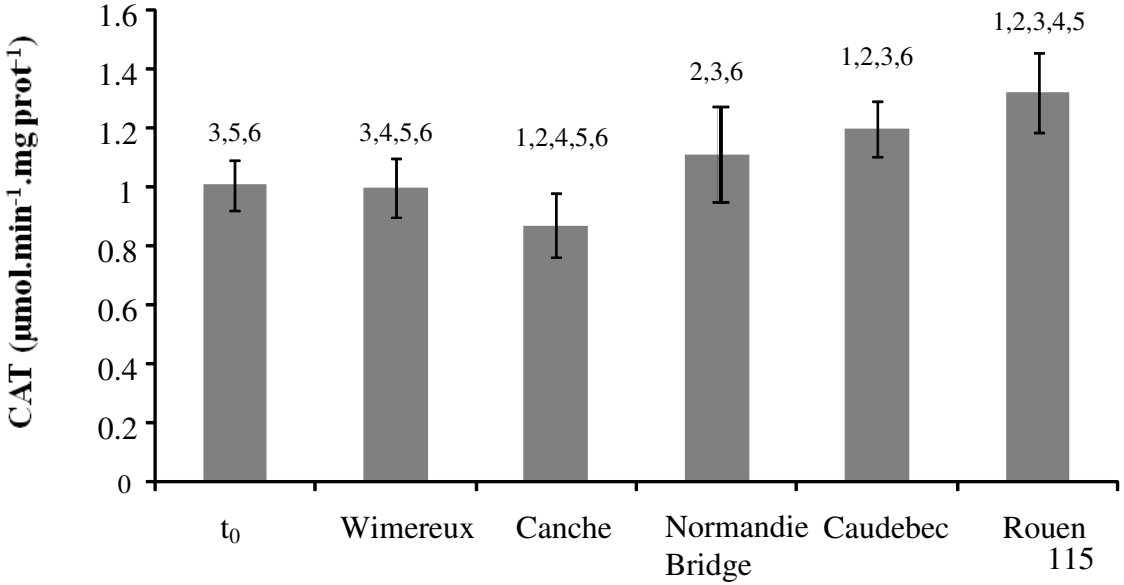




Figure 30. Biomarker measurements (EROD, GST and CAT) on sea bass juveniles at $t_7$. ([1]), ([2]), ([3]), ([4]), ([5]) and ([6]) represent significant difference compared to "$t_0$", "Wimereux", "Canche", "Normandie Bridge", "Caudebec" and "Rouen", respectively

III.2.3.5. Immune system responses

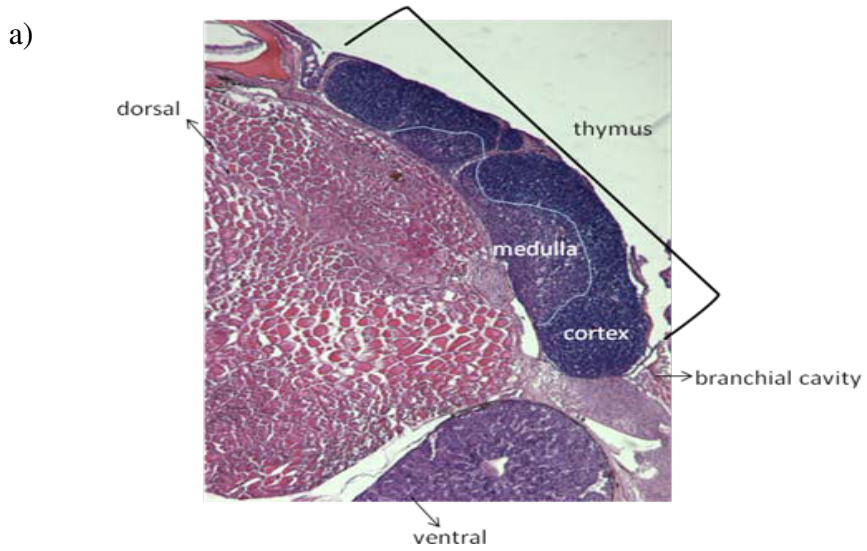
To determine the potential effects of contaminated sediments on the immature immune system of sea bass, two parameters, one dealing with the thymus development and the other monitoring the accumulation of melanomacrophage centers (MMCs) in spleen, were taken into account. The transversal section through the anterior section of *D. labrax* head shows the regional distribution of the thymus into cortex and medulla (Figure 31a).

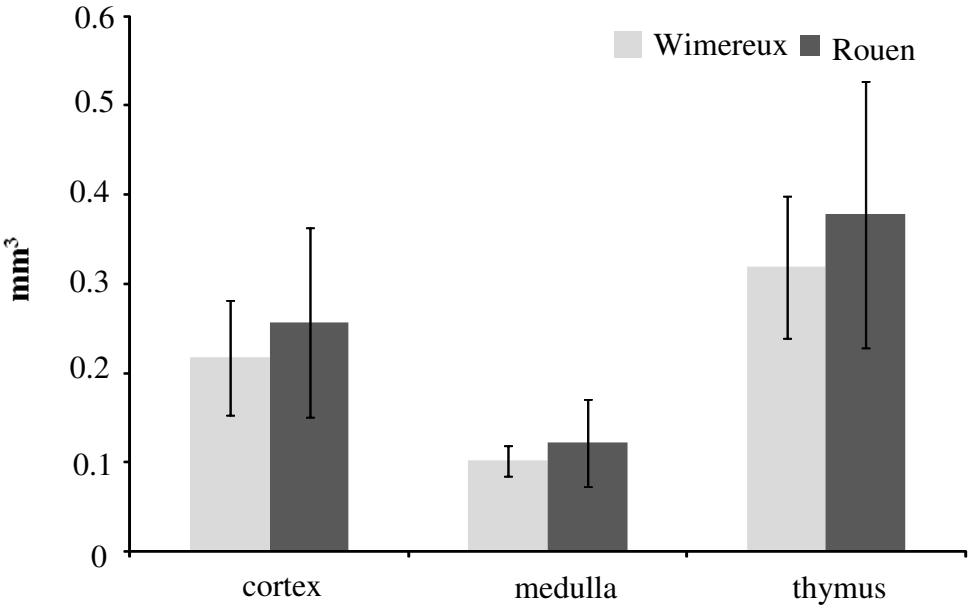
No significant differences in thymus volume or the thymic compartments volumes, cortex and medulla were revealed between control (Wimereux) and the most polluted sediment site (Rouen) (KW, $p>0.05$) (Figure 31b). Only a tendency for a lower individual variation in not contaminated fish for the thymus size could be observed. Comparing the thymus structure of exposed and non-exposed fish, no difference in the ratio of cortex and medulla (Wimereux: 0.497 ± 0.10; Rouen: 0.501 ± 0.07) could be identified. Even if the thymus volume is linked to the fish size (length, weight, height$^3$) there is no significant difference observable (Table 11).

No statistical difference in MMC accumulation could be detected between the control group and the group exposed to the most polluted site, but a tendency for more MMCs in the spleen of exposed fish can be remarked (KW, $p>0.05$) (Figure 31c).

Table 11. Correlation between thymus volume and fish length, weight and height$^3$ for fish exposed to sediment from Wimereux and Rouen

| Site | thymus volume/ fish length [mm$^3$/mm] | thymus volume/ fish weight [mm$^3$/mg] | thymus volume/ fish height$^3$ [mm$^3$/mm$^3$] |
|---|---|---|---|
| Wimereux | 0.006 ± 0.001 | 0.157 ± 0.030 | 0.026 ± 0.055 |
| Rouen | 0.007 ± 0.002 | 0.190 ± 0.061 | 0.030 ± 0.011 |



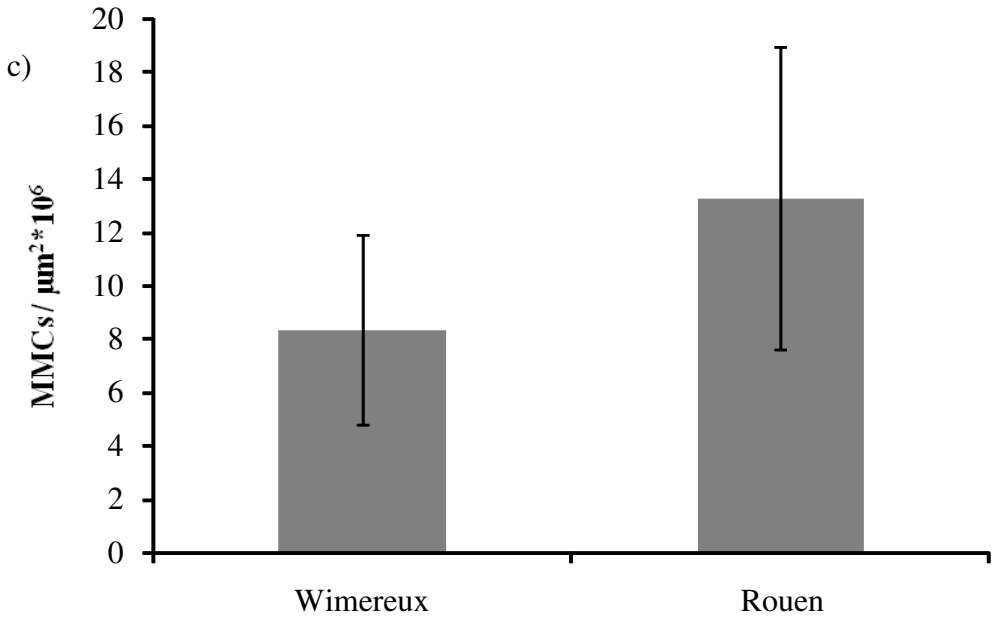




Figure 31. a) transversal section through the anterior section of *D. labrax* head (50x); the regionalization of the thymus into cortex and medulla is presented, b) the mean volume (±SD) of thymus, cortex and medulla is indicated in $mm^3$ for juvenile sea bass exposed to control sediment (Wimereux, n=5; light grey bars) and contaminated sediment (Rouen, n=5; dark grey bars), c) The number of melanomacrophage centers per area (MMC/$\mu m^2*10^{-6}$) in sea bass spleen exposed to control sediment (Wimereux; n=5) and contaminated sediment (Rouen; n=5); bars show mean with SD

III.2.3.6. Correlation between parameters

In order to integrate the overall measurement of biomarker activities, biological parameters and sediment chemical contaminants, a PCA analysis was performed (Figure 32). The two first axes of the PCA explained 91.37 % of the global inertia in the data with mean explanations for the first axis (78.34 %). The first axis ordinates the stations along a pollution gradient, which appears to be positively correlated with EROD activities. Growth in length (GL) and weight (GW) appear to be more associated with the second axis which seems to differentiate sea bass among the values of parameters. Among the different biological indicators, the RNA:DNA ratio appears to be the most related with biomarkers activities.

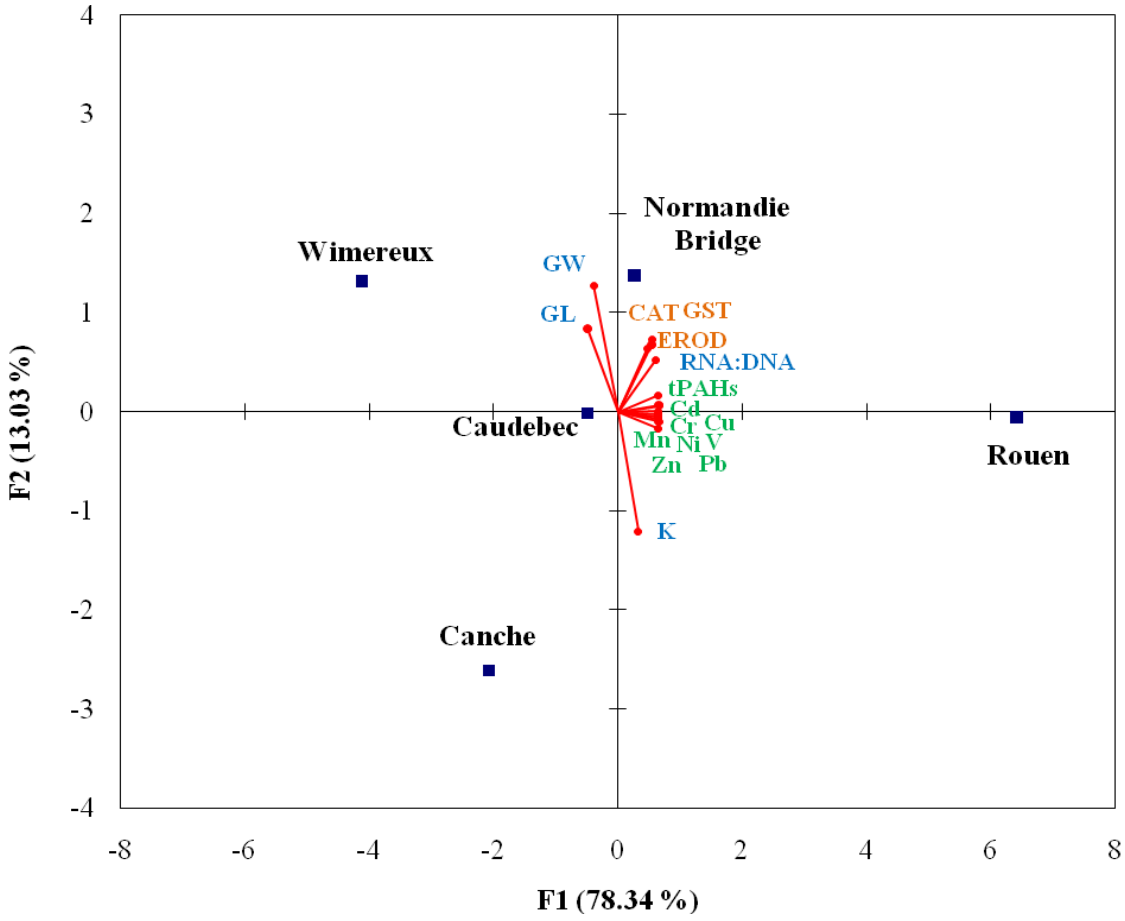



Figure 32. PCA based on biological parameters (specific growth rate in length (GL) and weight (GW), Fulton's condition index (K), RNA-DNA ratio) of juveniles sea bass in relation with the chemical contamination (metals, total PAHs) and biomarkers (EROD, GST and CAT) measured in the five sites

## III.2.4. Discussion

### III.2.4.1. Estuarine sediment contamination and metal accumulation in gills

The Seine estuary, considered to be one of the most impacted estuary in Europe, has received high inputs of a large variety of pollutants including PAHs, PCBs and metals that comes primarily from the upper part of the estuary due to the industrial activities and high urbanization of the Paris region (12 million inhabitants) (Carpentier et al., 2002; Meybeck et al., 2004; Dauvin, 2008). In this estuary, the concentrations of sediment contaminants increase from the Seine Bay to the upper part of the Seine estuary (Cachot et al., 2006). The chemical characterization of sediments sampled in the different sites in the Seine estuary proofs the presence of substantial and diverse contaminants, namely metals and PAH with anthropogenic origin. Our results showed that different levels of metals and PAHs contamination measured in the three Seine estuary sediment sites increased through the upper sampling zones. Although, no PCB was found in all sites, Wimereux was the lowest contaminated site and Rouen the highest. The chemical levels of concentration measured in the same Seine estuary sediments sites showed similar values as reported in Cachot et al. (2006).

In addition to anthropogenic impact, differences in metal contamination between the sites could be related with sediment characteristics while metals are known to have a high affinity for sediment organic matter and fine fraction (Ujević et al., 2000). This can explain that the less contaminated site, Wimereux, was characterised by sandy sediment and showed low organic matter content whereas other sites showed muddy sediment character.

In the present study, the accumulation of some metals (Cr, Cu, Pb, V and Se) in gills was higher in Rouen than other sites. Concentrations on Cd, Cu and Pb in gills were quite similar while Zn metal levels were higher as those measured in wild sea bass (< 15 cm) by Miramand et al. (2001) in the Seine estuary (between Honfleur and Tancarville Bridge). Although the high contents in Cd, Cu, Mn, Pb and Zn observed in sediments from the Seine estuary sites and their bioavailability similar to Cr, Ni and V, a direct relationship between metal estimated as bioavailable and metal bioaccumulated cannot be proofed. The suitability of fish gills analyses for environmental biomonitoring was recommended by several recent works (Fernandes et al., 2008; Dautremepuits et al., 2009; Oliveira et al., 2009; Pereira et al., 2009). All the analysed elements have toxic effects on biota above certain concentrations. They differ fundamentally in function and regulation in aquatic organisms. Some heavy

metals like copper, zinc and manganese are essential for fish metabolism since they play an important role in biological systems as Cu is a component of numerous oxidation-reduction enzyme systems and Zn is a component or cofactor in many important enzyme systems. Both are efficiently regulated by aquatic organisms, particularly fish (Amiard et al., 1987; Vallee and Auld, 1990; Beinert, 1996). Gills are the first organ to be in contact with water and resuspended sediment particles, so they can be relevant sites of interaction with metal ions. Indeed, the concentrations of essential metals in organisms tend to be highly regulated compared to nonessential. Fish, in particular, can use distinct strategies of metal homeostasis to achieve a steady-state balance: reducing metal accumulation and toxicity including uptake inhibition, increased elimination and detoxification, and storage (Fernandes et al., 2007).

### III.2.4.2. Physiological indicators

Measures of growth and condition indices of young fishes have been used to assess the effects of environmental conditions on individuals (e.g. Suthers et al. 1992). These indices reflect the habitat in which the fish live by using short-and mid-term indices (RNA/DNA ratio; recent growth index).

Condition indices are valuable tools to assess nutrition status and growth rates of juveniles. In previous studies, consistent differences in growth, condition indices and abundance of 0-group sole among sites that present various degrees of contamination within the English Channel and the Bay of Biscay were shown: juvenile sole caught in nurseries located near harbour and close to, or within, polluted estuaries presented lower growth, lower condition indices and lower abundances (Gilliers et al., 2006; Amara et al., 2007). Sites highly impacted by anthropogenic disturbances in the form of contamination by heavy metals and organic contaminants were shown to provide low quality habitats for juvenile fishes (Whitfield and Elliott, 2002) with consequences on fish growth and survival and population renewal (Gibson, 1994). Moreover, metals are of great interest as they can result in direct energetic and physiological costs, expressed in decreased growth and low physiological condition, as well as indirect costs due to the many defence mechanisms triggered (Livingstone, 2001; Marchand et al., 2003; van der Oost et al., 2003; Fonseca et al., 2009).

Although, growth rate in length and weight values were not significantly different between conditions, they are comparable with the literature. Values of specific growth in weight of sea bass in all sites are slightly lower to those measured on sea bass with the same initial weight by Eroldogan et al. (2004), and Conides and Glamuzina, (2006). Fonseca et al.

(2009) reported lower growth rate in weight than our values. Several studies have showed that chemical contaminant could reduce the fish growth for many species and particularly early life stages (Hopkins et al., 2004; Saborido-Rey et al., 2007; Abdel-Tawwab et al., 2007). Fulton's K condition index has often been used in stress assessment studies because this type of index is based on easily obtained length and weight measurements (Gilliers et al., 2006; Fonsceca et al., 2009). Despite the different sediment contamination levels, sea bass juveniles show a good condition in all sites compared to $t_0$. Nevertheless, condition values observed in the present study were comparable to those reported for *Solea senegalensis* and *Dicentrarchus labrax* by Foncesa et al. (2009, 2011) and Vasconcelos et al. (2009). Nucleic acid based indices, namely RNA content or the ratio of RNA to DNA content (RNA-DNA), have been used in numerous studies as indices for nutritional condition and growth assessment in juvenile fish (e.g. Fukuda et al., 2001; Gilliers et al., 2006) and has been widely applied to laboratory-reared as well as wild fishes (Clemmesen, 1988 ; Buckley et al., 1999). No differences were observed for sea bass juveniles among the five sites for these indices. Values of the RNA-DNA ratios of sea bass from all sites are lower with those measured by authors on the same species (Fonseca et al., 2009; Vasconcelos et al., 2009). Moreover, lower RNA-DNA values in fish were previously recorded from highly contaminated estuary (Humphrey et al., 2007; Vinagre et al., 2008b; Amara et al., 2009).

### III.2.4.3. Biomarker responses

Aquatic organisms are exposed to a wide variety of environmental contaminants and fish are generally considered as good model organisms for monitoring of the aquatic environment since they are very susceptible to environmental pollutants. Hence, a set of biomarkers such as detoxification and antioxidant enzymes in fish have been commonly used as an early warning indicators of chemical exposure (Van der Oost et al., 2003). Previous studies used individually some fishes (*Dicentrarchus labrax*, *Solea senegelansis* and *Pomatoschistus microps*) considering biomarker responses to contamination, in both laboratory (e.g. Gravato and Santos, 2003a, b; Fonseca et al., 2009; Vieira et al., 2009) and field conditions (e.g. Fernandes et al., 2007; Monteiro et al., 2007; Costa et al., 2009). Sanchez et al. (2005, 2006) examined a set of hepatic biotransformation and oxidative stress endpoints as potential biomarkers for various pollutants in stickleback. In this study, sea bass juveniles evidenced complex biomarkers responses to sediment contamination, exhibiting

differences at the biomarker activity level among different sites. We found significantly higher EROD, GST and CAT activities in the most contaminated site, Rouen; Normandie Bridge and Caudebec, respectively compared to control site, Wimereux.

As a biomarker EROD activity has been applied in numerous field investigations and laboratory studies, *e.g.* studies of bleached kraft mill effluents (BKME) (Soimasuo et al., 1995; Vandenheuvel et al., 1995; Karels et al., 1998), contaminated sediments (Engwall et al., 1996; Förlin et al., 1996) and oil spills and petroleum leaks (Jewett et al., 2002; Lee and Anderson, 2005b; Morales-Caselles et al., 2006) as well as in monitoring of general contamination (Kirby et al., 2004; Miller et al., 2004; Hansson et al., 2006). Gravato and Santos (2003a, b) and Teles et al. (2004) measured in sea bass juveniles same range of EROD activities as reported in this study. Schmitt (2002) and Schmitt et al. (2005) reported higher hepatic EROD activity (0.14 to 2.68 pmol/ min/mg) in pike from the Mississippi River and Rio Grande basins compared to our results. Several investigators have found EROD to be well correlated with the organic pollution gradient (Förlin et al., 1984; Andersson and Förlin, 1992; Stegeman et al., 1992; Bucheli and Fent, 1995; Whyte et al., 2000). In this study, EROD activity had a significantly positive correlation with all metals concentrations in the sediment, suggesting that these metals increase the reactive oxygen species (ROS) levels in this fish species and the antioxidant system responds to an increase of environmental stress. Maria et al. (2009) described lower antioxidant enzyme activities in sea bass from Ria de Aveiro in October 2005, yet they associated this low response to a significant inhibition of the antioxidant system by the complex mixture of environmental contaminants in several estuarine sites. Another study considering wild sea bass biomarker responses from the south coast of Portugal described an antioxidant enzyme activity 100 fold higher than the present study (Fernandes et al., 2007). Regarding biotransformation enzymes, and assuming that EROD activity is a specific biomarker of PAH-like compounds exposure, it would be excepted that individuals from Rouen had higher EROD activity compared to control, Wimereux, given that Rouen sediments contained higher PAH concentration. Using a single i.p. injection of PAH (20 mg kg-l wet weight), hepatic EROD activity of *D. labrax* increased 7- to 24-fold with exposure to 3MC compared with 5- to 16-fold with exposure to benzo[a]pyrene (BaP) (Lemaire et al., 1992a, b).

Strong GST inhibition was observed in sea bass juveniles where the highest metals and PAHs concentrations were measured in the present study. Our results of GST activities were in the same range of values than those reported by Fernandes et al. (2007) and Kopecka

Pempkowiak (2008). Previous studies have also described a bell-shaped pattern in GST activity for different species, where the enzyme activity increases with increasing contaminant exposure until a certain concentration where enzymatic activity progressively decreases (Elia et al., 2003; Vieira et al., 2009). Moreover, both GST inhibition and induction after exposure to different PAH and metals has been reported (Sanchez et al., 2005; Vieira et al., 2008). The usefulness of measuring the induction of GST activity as a biomarker of exposure to xenobiotics was demonstrated previously in several laboratory and environmental studies. The results presented by Gubbins et al. (2000) showed that glutathione S-transferase was (together with CYP1A) induced in salmon following paralytic shellfish poisoning exposure. Porte et al. (2000) observed a higher level of GST activity in Mediterranean deep-sea fish influenced by urban and industrial waste water. The induction of GST activity in flounder exposed to organic contaminants (e.g., PAH, PCB) in Norwegian fiords has been reported by Beyer et al. (1996). An increase in hepatic GST activity has been reported in several studies after exposure of various fish species to PAHs, PCBs, OCPs and PCDDs, but most studies did not demonstrate any significant alterations (e.g. Collier et al., 1992; Vigano et al., 1995; Lemaire et al., 1996). Attempts to detect chemically induced activities of GSTs in free living fish also yielded conflicting results. Several studies reported GST activities to be significantly increased, but in most cases no significant differences were observed between fish from control and polluted sites (Van der Oost et al., 2003). A significant decrease in GST activities was observed in rainbow trout, sea bass, seabream and sunfish exposed to PCDDs, pesticides or PAHs (e.g. Hektoen et al., 1994; Oikari and Jimenez, 1992), and in some fish species in polluted environments (e.g. Bagnasco et al., 1991; Otto et al., 1996; Tuvikene et al., 1999).

Most organisms have antioxidant defenses against ROS produced during aerobic respiration (Scandalios, 2002) or by exposure to xenobiotics (Livingstone et al., 1993). Catalase, which protects tissues against damage by ROS, was one of the first enzymes proposed to be an effective marker of oxidative stress. Our results indicated higher CAT activities in Seine estuary stations and a significant inhibition in Canche estuary compared to control, Wimereux. In this study, the values of CAT activities were comparable with those reported by Kerambrun et al. (2011) and 3 fold higher than those of Lemaire et al. (1996) in the same species. Various responses of CAT activity have been observed in animals exposed to organic or metallic contaminants in both field and laboratory experiments and CAT has been shown to be either induced or inhibited by metals depending on the dose, the species or the route of exposure (Romeo et al., 2000; Sanchez et al., 2005). Hence, elevated hepatic

CAT activities were observed in experiments with fish exposed to PCBs- or PAH-containing sediments, but other laboratory studies failed to demonstrate any significant CAT changes. Both induction and inhibition were observed after the exposure of fish to environmental pollution (Van der Oost et al., 2003). CAT has been Zebrafish (*Brachydanio rerio*) exposed to 40 and 140 $mgCuL^{-1}$ as CuSO4 presented an induction of hepatic CAT within 2 weeks of waterborne exposure (Paris-Palacios et al., 2000). Conversely, in carp (*Cyprinus carpio*), hepatic CAT was inhibited after 96 h exposure to 100 and 250 $mgCuL^{-1}$ as CuSO4 (Dautremepuits et al., 2002). An inhibition of CAT activity in the liver of rainbow trout (*Oncorhyncus mykiss*) and killifish (*Fundulus heteroclitus*) exposed to Cd has also been reported (Pruell and Engelhardt, 1980; Palace et al., 1992). Increases in hepatic CAT activity were only observed in some experiments with fish exposed to PCBs, BKME or PAH-containing sediments, but most laboratory studies could not demonstrate any significant alterations (e.g. Di Giulio et al., 1989a, b; Rudneva-Titova and Zherko, 1994).

### III.2.4.4. Immune system alterations

The examination of thymus and spleen from juvenile sea bass gives a clue about the effects of polluted sediments on the functionality of both parts of the immature fish immune system, e.g. innate and adaptive immunity.

The thymus is known as primary lymphoid organ in fish, where the maturation of the T-lymphocytes takes place (Picchietti et al., 2008, Fishelson, 2006) and is therefore part of the adaptive immune system. Separated only through an epithelial cell layer from the gill chamber the thymus is like gills a tissue sensitive to waterborne pollutants (Press and Evensen, 1999). The thymus gland is said to reach adult size 365 dph (Abelli et al., 1996). In opposition to mammals there is no thymic atrophy observable in adult fish. The organ elaborates and provides T-lymphocytes during whole fish life (Fischelson, 2006). Therefore chronic effect of pollutants on the adaptive immunity can be indicated by thymic atrophy (Grinwis et al., 1998; Fishelson, 2006).

The organ is parted into cortex and medulla. While the cortex is the compartment of immature T-lymphocytes, mature T-cells could be observed in the medulla. Picchietti et al. (2009) suggest the critical period for thymic regionalization between 51 and 92 dph in sea bass development.

The present exposure study indicates no difference in thymus volume or its compartments between the fish exposed to the highest contaminated sediment and the control fish. No thymotoxicity during 21 day exposure to the Rouen sediment could be observed. As shown by Grinwis et al. (1998, 2009) and Wester et al. (1985, 1986, 2002) toxic effects on thymus development seems to be species- and chemical-dependent. While tributyltinoxide (TBTO) has an effect on thymus in rat, guppies and to a lesser extend in flounder (20% of relative thymus volume), no effect was observed in medaka. Further studies with flounder indicate an impairment of thymus volume induced by 2.3.7.8-tetrachlorodibenzo-p-dioxion (TCDD) and PCB-126 (Grinwis et al., 2000b). But for these experiments the exposure time was nearly four times longer than for the experiment described above. The observed tendency of a higher individual variation for juvenile sea bass challenged with the Rouen sediment might indicate an impairment after a longer exposure time as demonstrated for TCDD in flounder (Grinwis et al., 2000a, Wester and Canton, 1987).

In contrast to the thymus, the spleen is classed as secondary lymphoid tissue with additional hematopoietic function. The high number of macrophages found in spleen indicates an elevated phagocytotic activity in this gland, especially for decomposed blood cells or particles (Fiselsen, 2006). This is also reflected by the occurrence of macrophage aggregations, so called melanomacrophage centers (MMCs). These are described as agglomerations of phagocytoting macrophages supplemented with pigments, mostly melanin, and are recognized as important for immune functions in fishes (Blazer et al., 1997). Generally, the number of MMCs is suggested to increase due to environmental stress like deoxygenation or iatrogenic chemical pollution (Grinwis et al. 1998; Agius and Roberts, 2003). Fournie et al. (2001) emphasized that only densities of MMCs above 40/mm² can be correlated with high sediment contaminants and low dissolved oxygen. However the latter condition was not fulfilled in the conducted experiment. Generally it needs to be said that the number of MMCs observed per area is very low compared to literature (Fishelsen, 2006), but actually it is also stated that the number of MMCs in spleen increases with fish age and therefore with the number of pathogen and pollutant challenges.

Summarizing, during this experimental conditions no impairment of sediment released pollutants on chosen immune parameters could be detected.

III.2.5. Conclusion

A multi-biomarker approach to aquatic environmental monitoring allows the assessment of whole animal response to a range of anthropogenic contaminants and to predict the potential environmental impact of novel contaminants. In addition, a holistic approach to monitoring of this type will provide a greater insight into the actual effects of contaminants from the sub-cellular to ecosystem levels. The current study supports the utility of European sea bass as a sentinel species for the measurement of sediment contamination on the physiological performances linked to biochemical biomarkers and immune system responses. Measures such as detoxification (EROD, GST) and oxidative stress enzymes (CAT) may provide a useful tool when considering short-term exposures to chemicals with other general immunological parameters such as the accumulation of melanomacrophage centers (MMC) used for stress in fishes. Following these responses, the use of physiological biomarkers, such as growth and condition indices are also complementary tools to assess sediment contamination and so habitat quality on juvenile fish.

Overall, selection of appropriate species for monitoring habitat quality through assessment of contaminant-induced effects is fundamental, and despite the added logistical difficulties, the value of a multi-specific approach is unquestionable for an ecosystem-based approach.

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# CHAPTER IV

# EFFECTS OF ALGAL BLOOM

# CHAPTER IV - 1

# EFFECTS OF TRANSPARENT EXOPOLYMER PARTICLES (TEP) DERIVED FROM *Phaeocystis globosa* BLOOM ON THE PHYSIOLOGICAL PERFORMANCE OF EUROPEAN SEA BASS JUVENILES

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**ABSTRACT**

The aim of this study is to analyse the effect of exudates and transparent exopolymeric particles (TEP) produced from *P. globosa* bloom senescence on the physiological performance and mortality of European sea bass juveniles. Thus, during a 28 days mesocosm experiment (1 $m^3$ enclosures), sea bass juveniles (113 days old) were exposed to high TEP concentrations derived from decaying *P. globosa* colonies (7634.27 ± 3514.41 µg XG eq $L^{-1}$) and foam (932.54 ± 341.21 µg XG eq $L^{-1}$). Environmental parameters (pH, dissolved oxygen, temperature, and salinity) were stable, similar among treatments and not limiting for growth. Thus, only TEP concentration varied between treatments. Fish mortality rate was relatively low (0 to 2 % $d^{-1}$) and occurred mostly during the first week when TEP concentrations were low. Biological performances (growth in length and weight, Fulton condition index, and RNA-DNA ratio) of juvenile sea bass were generally similar both between replicates and conditions. During the 28 days of the experiment, juvenile sea bass size increased by 7.2 mm (0.26 $mm.d^{-1}$) and the weight by 708 mg (25.29 $mg. d^{-1}$). The results obtained clearly showed that exudates and TEP excreted from decaying *P. globosa* bloom and foam accumulation have no negative effect on juvenile sea bass physiological performance and survival and hence on their recruitment success.

**Keywords:** Sea bass, *Phaeocystis globosa*, TEP, sea foam, growth, condition index

## IV.1.1. Introduction

For many marine species, including commercial fish species, shallow coastal marine habitats serve as spawning and nursery grounds. Sometimes referred in the literature as "essential fish habitats", these habitats are thought to provide ecological advantages (Beck et al., 2001). However, these attractive ecosystems are typically submitted to a variety of stressors, both natural and anthropogenic, which may alter their ecological functions (Meng et al., 2001; Amara et al., 2007). Indeed, coastal areas around the world suffer a variety of environmental problems, including loss or destruction of habitats and decline of coastal waters quality. Among the stressors affecting the ecology of coastal areas, High Biomass Harmful Algal Blooms (HB-HABs) have become a significant worldwide threat to fisheries, public health, and economy. HB-HABs have increased in frequency, duration, and distribution in recent decades (Treasurer et al., 2003; Tang and Gobler, 2008) and most of them have been linked to eutrophication (Masó and Garcés, 2006).

HB-HABs result from the dominance of a particular phytoplankton species or group of species that can be considered directly or indirectly toxic or non-toxic for humans and marine organisms (Bruslé, 1995; Masó and Garcés, 2006). These blooms may be noxious for aquatic species and particularly for marine and freshwater fishes, either causing anoxia due to inefficient degradation of the accumulated algal biomass and clogging of fish gills, or producing specific toxins which are responsible for acute mortalities of wild and farmed fish worldwide (Bruslé, 1995). Larval and juvenile fish can also suffer from these HB-HABs. For instance, Potts and Edwards (1987) reported a drastic reduction in young fish off the coast of Plymouth following a bloom, which adversely affected the surrounding area.

*Phaeocystis* spp. is one of the most recurrent phytoplankton blooms recorded in the northwest European shelf seas and can represent 80 % of total phytoplankton abundance in spring (e.g. Breton et al., 2000; Breton et al., 2006; Schapira et al., 2008; Grattepanche et al., 2011). The life cycle of *Phaeocystis* involves the alternation between solitary cells of few micrometers and mucilaginous colonies of up to several centimeters in size (Rousseau et al., 1994; Chen et al., 2002). This size spectra shift throughout *P. globosa* bloom development is acknowledged as a defence strategy against predation and one of *P. globosa* way of success (Veldhuis, 2005) as it creates a size mismatch problem for small grazers (Weisse et al., 1994). Another consequence of the formation of gel-like *Phaeocystis* colonies is the potential effects of phytoplankton-derived polymeric materials on seawater properties such as viscosity (Seuront et al., 2006; Seuront and Vincent, 2008) and transparency. Colony proliferation

affects the penetration of light in the water column, thus seriously impacting on the abundance, metabolism, growth, feeding and behaviour of marine organisms (Dauvin et al., 2008; Spilmont et al., 2009).

The wane of the *Phaeocystis* bloom also coincides with high concentration of Transparent Exopolymeric Particles (TEP; Alldredge et al., 1993) formed from the coagulation of *Phaeocystis* exudates (acidic polysaccharides) and, in major part, from the release of large fragments of the mucilaginous colonial matrix (Alldredge et al., 1993; Passow and Alldredge, 1995b; Passow, 2002a, b; Mari et al., 2005). This accumulation of mucilaginous aggregates on the sea surface strongly depends on wind conditions (Seuront and Souissi, 2002). Consequently, beaches are often covered with an impressive layer of foam (Lancelot, 1995). The water can then resemble fresh white of egg (Dreyfuss, 1962) and this often leads to clogging of plankton and fishing nets (Chang, 1984). The impact of foam formation has mainly been studied on bacterio- and zooplankton population (Becquevort et al., 1998; Seuront and Vincent, 2008). Although foam accumulation can strongly affect macrobenthic fauna diversity and trigger drastic mortality (Desroy and Denis, 2004; Peperzak and Poelman, 2008).

Additionally, TEP produced via colony disruption are resistant to microbial breakdown (Janse et al., 1999). Therefore, it is likely that a high proportion of this refractory fraction leads to the formation of settling marine snow and/or enters the plankton food web. TEP may serve as food for several organisms (Passow and Alldredge, 1999; Ling and Alldredge, 2003). However, negative impact of TEP on zooplankton feeding (Prieto et al., 2001; Dutz et al., 2005) is often reported and attributed to the inhibitory effect of phytoplankton excreted exudates (e.g. carbohydrates; Liu and Buskey, 2000; Dutz et al., 2005). *Phaeocystis* has also been suspected to be itchthyotoxic (Aanesen et al., 1998; Stabell et al., 1999; Chen et al., 2002). Since avoidance strategies were recorded for fishes in natural environments during *Phaeocystis* blooms (Savage 1930; Rogers and Lockwood, 2002). When escaping is not possible, exposure to *Phaeocystis* bloom can have deleterious effects. For instance, chemical compounds produced by *Phaeocystis* were reported to be lethal to cod larvae (Aanesen et al., 1998) as well as to caged fish along the Chinese coasts (Huang et al. 1999; Chen et al., 2002). *Phaeocystis* was also shown to impact on fish physiology, declining the appetite of cage-reared Atlantic salmon (Eilertsen and Raa, 1995). Other trophic effects linked to colony size (e.g. Weisse et al., 1994), toxic peculiarities (e.g. acrylic acid, DMSP; Sieburth, 1960; Estep et al., 1990) or mechanical hindrance (e.g. clogging of feeding

appendages; Schnack et al., 1985) and biologically induced modification of seawater viscosity (Seuront et al., 2006) have all been suggested.

Although high biomass blooms of *Phaeocystis* may cause serious ecological and economical problems with their harmful effects on the environment and biota (Lancelot et al., 1987; Masó and Garcés, 2006) there is no information on their effects on fish physiological performances, health or mortality. The hypothesis of this study is that exudates and TEP excreted from decaying *P. globosa* bloom have the potential to negatively affect juvenile fish in the shallow coastal zone (e.g. nursery areas). This hypothesis was tested by carrying out a replicated mesocosm experiment over 28 days. In our experiment, *P. globosa* grown at late stationary phase along with fresh foam accumulated at seawater surface were used as TEP sources and regularly provided to sea bass juvenile fish *(Dicentrarchus labrax)*. Mortality and physiological performances were assessed and results are discussed with regards to recurrent bloom development and their potential impact on fish nursery grounds.

## IV.1.2. Materials and Methods

### IV.1.2.1. TEP production from decaying algal cultures and foam

*Phaeocystis globosa* strain was isolated from the eastern English Channel in autumn 2008 pippeting cells from coastal water samples. *P. globosa* was maintained in monospecific conditions in f/2 medium (Guillard and Ryther 1962; Guillard, 1975) at 12 ± 0.5°C with a 12:12 h light:dark cycle under a photon flux density of 400 µmol photons $m^{-2}$ $s^{-1}$ (Daylight HQIT-WD 250 W F, OSRAM). Prior to the experiment, *P. globosa* was grown for 2 weeks under the same light conditions in a 300L plexiglass batch filled with UV sterilized seawater at 15.0 ± 0.5°C. This time delay assured *P. globosa* to be in late stationary phase, TEP concentration being thus comparable or higher to observed values during *Phaeocystis* blooms (8 to 12 mg eq X $L^{-1}$; Mari et al., 2005; Klein et al., 2011; E. Breton, pers. com.) and nutrients being nearly depleted.

Freshly accumulated foam and associated TEP were regularly collected at the hatchery pumping site and volume was assessed by gently placing the foam in a large bucket of known volume. Care was taken not to press the foam so as to preserve its natural aspect and its apparent density.

IV.1.2.2. Experimental set up and sampling strategy

In order to explore the effects of *P. globosa* derived material on fish survival and physiological performances, a mesocosm experiment was carried at Aquanord hatchery over 28 days in 2009 (April 17 to May 15). Six circular tanks of $1m^3$ each were supplied with sand-filtered and UV sterilized running seawater, thus permitting to keep mesocosm at *in situ* temperature (15 ±1°C) throughout the experiment. Tanks were illuminated according to the natural photoperiod at a photon density of 400 µmol $m^{-2}$ $s^{-1}$ (Daylight HQIT-WD 250 WF) and gently aerated (compressed air), thus preventing settling of particles and maintaining oxygen saturation above 80%.

Juvenile sea bass (1550 individuals) were obtained from the Aquanord hatchery and reared at low density (0.25 ind $L^{-1}$). At the beginning of the experiment ($t_0$), 50 individuals were randomly sampled, anesthetized for 3-4 minutes with 2-phenoxyethanol (0.32 mL $L^{-1}$ final concentration) and frozen (-20°C) for analyses of size, weight and condition.

250 individuals (aged 113 days old) were randomly distributed in the six tanks. Every 2 days, two tanks (*Ph1* and *Ph2*) received 50 L of *P. globosa* culture to maintain high and stable TEP concentration in each tank. Two other tanks (F1 and F2) received every 2 days 46L of freshly accumulated foam and its associated TEP. The last two tanks (C1 and C2) only contained juvenile sea bass and were used as controls. In each tank, 25% of the seawater was renewed every two days, except for C2 where continuous seawater flow of 500 L $min^{-1}$ was processed so as to mimic the water flow conditions generally encountered in the rearing tanks of the Aquanord hatchery.

Fish were fed daily *ad libitum* every morning with commercial dry pellets (Skretting Ltd., France, Gemma PG 1.0) which contain 56% protein, 18% oil, 10% dry ashes, 0.6% fibre, 1.3% total phosphorus, copper (8 ppm $CuSO_4$), vitamin A (15000 I.U. / kg), vitamin D3 (1125 I.U. / kg) and vitamin E (225 I.U. / kg).

Daily observations were carried out every morning before the first food supply to assess fish mortality. Sampling and measurements were always done at a 2 days frequency before seawater renewal and fish feeding. Dissolved oxygen concentration (DO, mg $L^{-1}$), seawater temperature (°C), salinity, pH using a Hanna HI 9828 multiprobe and turbidity (NTU) using a turbidimeter (Eutech instruments, TN-100) were measured. For TEP analyses, 40 mL seawater samples were preserved with formaldehyde (2% final concentration) and stored at 4°C in the dark until analysis (within one month). For fish growth and physiological performances monitoring, 30 individuals from each tank were removed on day 8 and day 20.

At the end of the experiment (day 28), all the remaining fishes were removed from the tank. After each removal, fishes were anaesthetised and frozen at -20 °C.

### IV.1.2.3. Determination of TEP concentrations

TEP concentrations were determined according to the colorimetric method of Thornton et al. (2007). Briefly, 12 ml of seawater preserved sample were first dialyzed for 24 h with 1000 Da Molecular Weight Cut Off (MWCO) to remove salts which may interfere with the binding properties of Alcian blue. The following day, dissolved Acid Polysaccharides (APS) were separated from TEP by filtering 7 ml of the dialyzed filtrate at low vacuum (< 0.1 bar) onto 0.2 µm polycarbonate Nuclepore filters. APS were then stained with 1 mL of Alcian Blue stock solution (0.02% Alcian blue in 0.06% acetic acid; pH adjusted to 2.5; Passow and Alldredge 1995a). Stained APS precipitates were retained on a syringe filter containing a surfactant free cellulose acetate (SFCA) membrane with a pore size of 0.2 µm (Nalgene). Finally, the amount of dye remaining in the filtrate was determined spectrophotometrically at 610 nm and was inversely proportional to APS concentrations. The remaining 5 ml dialyzed filtrate was directly stained with Alcian Blue to bind both dissolved APS and TEP and then filtered on SFCA syringe filter. TEP concentrations were deduced from the difference of absorbance between the pools [TEP+APS] and [APS] and expressed as Gum xanthan equivalent per liter (GX eq $L^{-1}$).

### IV.1.2.4. Fish mortality and physiological performance

Dead fishes were counted daily and instantaneous daily mortality coefficients (M, $d^{-1}$) were estimated for each condition and tank applying the exponential model of decline:

$$Nt = N_0 \exp^{-M\,(t-t0)}$$

where $N_t$ is the number of fishes at time $t$. $N_0$ is the number of fishes at the beginning of the experiment ($t_0$) , $(t-t_0)$ is the experiment duration (days), and M is the instantaneous daily mortality coefficient.

At the laboratory, morphometric measurements were made on a total of 1550 juveniles (50 for $t_0$, 30 per tank for $t_8$, $t_{20}$ and 190 per tank for $t_{28}$). Each juvenile was thawed at room temperature, weighed (W, fresh weight; mg) to the nearest 0.1 mg and measured for standard (SL, mm) and total (TL, mm) length to the nearest 0.5 mm. Standard length (SL) was

preferred to total length (TL) because a significant number of individuals had their caudal fins damaged.

Juvenile sea bass specific growth rates in weight (% $d^{-1}$) were estimated as:

$$GW = 100(ln\ W_t - ln\ W_0) / (t - t_0),$$

where $W_0$ and $W_t$ are fish total body weight at times $t_0$ and $t$ (time of collection), respectively. Similarly, the specific growth rate in length (% $d^{-1}$) was estimated as:

$$GL = 100(ln\ L_t - ln\ L_0) / (t - t_0),$$

where $L_0$ and $L_t$ are fish standard length at times $t_0$ and $t$, respectively.

Two condition indices were estimated during the experiment. Firstly, the RNA:DNA ratio as an indicator of growth and nutritional status, and, secondly, the Fulton's K condition index as an indicator of the general well-being. This latter morphometric index assumes that heavier fish for a given length are in better condition. Individual condition factor (K) was determined from morphometric data, according to the formula:

$$K = (100 \times W) / SL^3$$

where W is the body weight (mg) and SL is the standard length (mm).

The RNA-DNA ratio was calculated at time $t_0$ and $t_{28}$ for each incubation condition (25 individuals for $t_0$ and 15 individuals per tank for $t_{28}$).

Nucleic acid quantification and subsequent RNA–DNA ratios have been used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Gwack and Tanaka, 2001). This biochemical index reflects variations in growth-related protein synthesis, since the quantity of ribonucleic acid (RNA) varies with the rate of protein synthesis, while the amount of deoxyribonucleic acid (DNA) per cell is species-constant in somatic tissue (Buckley and Bullow, 1987). The procedure used to determine RNA and DNA concentrations in individual fish is based on the Clemmesen method (1988) slightly modified by Amara et al. (2009). However, heads and fins were discarded before analysing fish and guts were excised to ensure that gut contents did not contribute to RNA–DNA ratio. Fish muscle sample (0.05 g) was homogenized in ice-cold Tris–EDTA buffer (0.05 M Tris. 0.1 M NaCl. 0.01 M EDTA. pH 8.0) using an Ultraturrax and subsequently transferred to a mixture of Tris–EDTA buffer, proteinase-K (pro-K) and sodium dodecyl sulfate (SDS). Nucleic acids were extracted by purification steps involving phenol-chloroformisoamylalcohol (Amara et al., 2009). The quantity of RNA and DNA was determined by the fluorescence-photometric technique using a specific nucleic acid fluorescent dye-ethidium bromide (Sigma-Aldrich Chemicals. France). The fluorescence due to total RNA was calculated as the difference between total fluorescence (RNA and DNA)

and the fluorescence after RNAase treatment which is assumedly to be due to the presence of only DNA. Salmon sperm DNA (Sigma−Aldrich Chemicals, Fr ance) and yeast type III RNA (Sigma−Aldrich Chemicals. France) were used as standards. RNA and DNA contents are both expressed as µg per µL.

IV.1.2.5. Statistical analysis

Since physico-chemical and biological data considered here did not comply with the parametric assumption of normality and variance equality, non−parametric Kruskall−Wallis test followed by the post hoc Dunn test (joint ranking test) were used for pair wise comparisons. TEP concentrations in the different conditions (*Ph1, Ph2* and F1, F2) were analysed by Mann-Whitney test. A significance level of a minimum of 5 % was considered in all statistical analyses. The statistics were performed using XLSTAT software package (version 5.01).

IV.1.3. Results

IV.1.3.1. Physico-chemical variables

Except for temperature, all measured physico-chemical parameters (salinity, DO, pH, turbidity) were stable over the 28 days of experiment. Water temperature showed similar trends over the study, increasing the first two days from 13°C to 15°C and then stabilizing to 15-16°C until the end of the experiment. Results from C2 treatment revealed significantly lower mean temperature (14.22 ± 0.42 °C), DO (9.52 ± 1.01 mg $L^{-1}$), and turbidity (0.49 ± 0.26 NTU) and significantly higher mean pH (8.25 ± 0.08) than the other treatments (Table 12; KW, $p<0.001$). In contrast, there was no significant differences in mean salinity of C2 (33.01 ± 0.24) compared to the other treatments (KW, $p=0.294$). These discrepancies may result from continuous water flow conditions experienced in tank C2 while other treatments were submitted to seawater renewal.

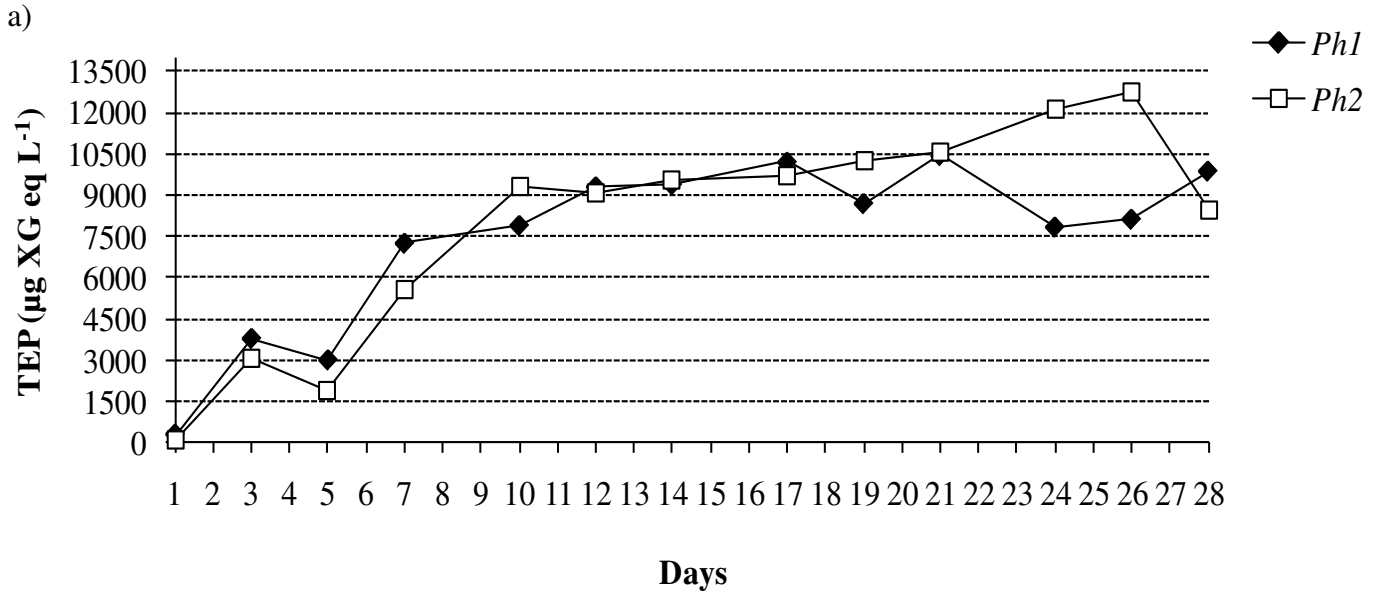
Table 12. Environmental context of the mesocosm experiment. Mean values (± SD) of the physico-chemical parameters measured (temperature, salinity, dissolved oxygen, pH and turbidity) over the course of the experiment (17 April to 15 May) in Control, *Phaeocystis* and Foam treatments. Values in parentheses stand for the range ($X_{min}$-$X_{max}$) of each parameter

| Treatment | Temperature (°C) | Salinity | Dissolved oxygen (mg $L^{-1}$) | pH | Turbidity (NTU) |
|---|---|---|---|---|---|
| C1 | 15.21 ± 0.60 (13.58 – 15.89) | 32.74 ± 0.58 (30.95 – 33.19) | 12.19 ±1.62 (10.14 - 14.67) | 7.86 ± 0.15 (7.53 – 8.09) | 0.97 ± 0.53 (0.09 – 1.90) |
| C2 | 14.22 ± 0.42 (13.53 - 14.71) | 33.01 ± 0.24 (32.33 - 33.23) | 9.52 ± 1.01 (7.70 -11.22) | 8.25 ± 0.08 (8.08 - 8.36) | 0.49 ± 0.26 (0.19 - 0.90) |
| *Ph1* | 15.15 ± 0.64 (13.34 – 15.79) | 32.94 ± 0.12 (32.73 -33.11) | 14.31 ± 2.47 (10.23 – 18.68) | 7.88 ± 0.12 (7.59 – 8.03) | 0.84 ± 0.55 (0.29 – 2.10) |
| *Ph2* | 15.18 ± 0.64 (13.83 – 15.88) | 32.92 ± 0.15 (32.65 – 33.17) | 13.48 ± 1.98 (9.94 – 16.13) | 7.87 ± 0.10 (7.66 – 8.05) | 1.02 ± 0.62 (0.29 – 2.05) |
| F1 | 15.03 ± 0.60 (13.43 – 15.70) | 32.91 ± 0.19 (32.50 – 33.21) | 16.06 ± 3.10 (10.38 – 21.38) | 7.85 ± 0.12 (7.56 – 8.02) | 1.23 ± 0.59 (0.64 – 2.52) |
| F2 | 15.09 ± 0.56 (13.66 – 15.83) | 32.94 ± 0.14 (32.67 – 33.15) | 14.33 ± 2.75 (11.84 – 21.66) | 7.85 ± 0.13 (7.53 – 8.10) | 1.35 ± 0.61 (0.38 – 2.32) |

IV.1.3.2. TEP concentrations

Over the experiment, TEP concentrations originating from decaying *P. globosa* cultures (*Ph1* and *Ph2*) increased by a factor 40 to 120. Concentrations ranged from 261 to 10457 µg XG eq $L^{-1}$ and from 70 to 12 734 µg XG eq $L^{-1}$, for *Ph1* and *Ph2*, respectively (Figure 33a). Mean TEP concentrations were not significantly different between tanks reaching 7388 ± 3128 µg XG eq $L^{-1}$ in *Ph1* and 7881 ± 3977 µg XG eq $L^{-1}$ in *Ph2* (M-W, p=0.418). TEP produced from *P. globosa* foam (F1 and F2) showed 8 fold lower concentrations than for decaying colonies and concentrations increased slightly over the course of the experiment ranging from 322 to 1551 and from 301 to 1521 µg XG eq $L^{-1}$ in F1 and F2, respectively (Figure 33b). No significant difference in mean TEP concentrations was observed between F1 (935 ± 353 µg XG eq $L^{-1}$) and F2 (930 ± 343 µg XG eq $L^{-1}$; M-W, p=0.880).

a)




b)

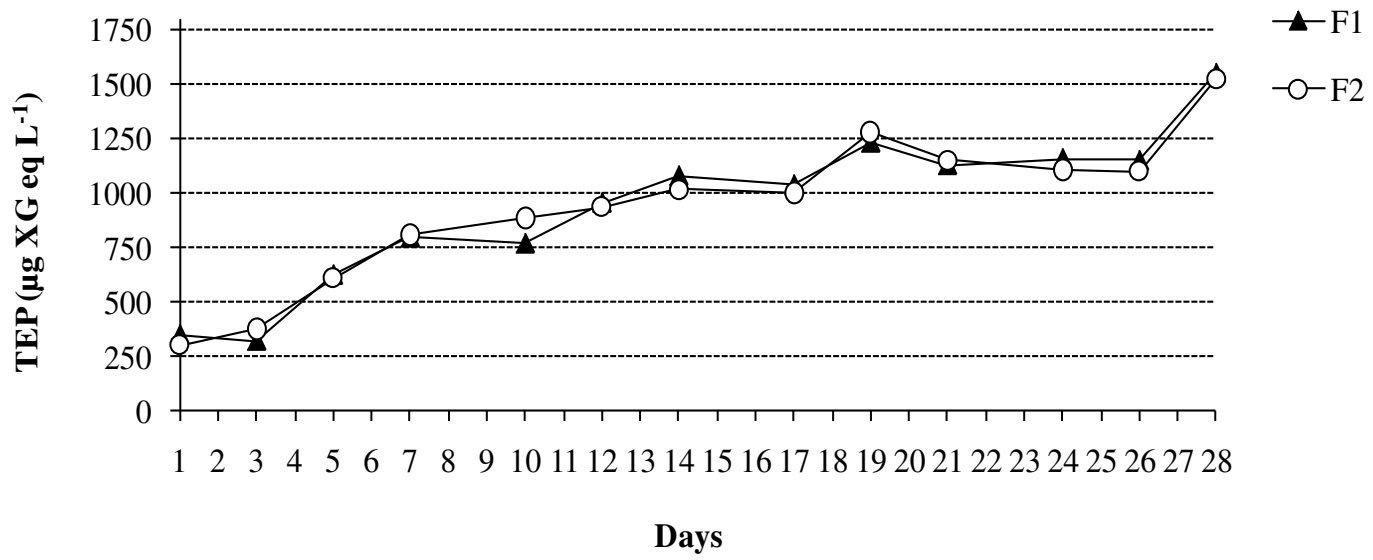



Figure 33. Concentration of transparent exopolymeric particles (TEP, in µg GX eq $L^{-1}$) in tanks containing decaying *Phaeocystis* colonies (*Ph1* and *Ph2*; a) and Foam (F1 and F2; b) during the experiment

IV.1.3.3. Fish mortality and physiological performance

No fish malformation, morphological abnormalities or lesions indicating cannibalism were observed during the course of the experiment. Mortality rate was relatively low and varied between 0 and 2.05 % $d^{-1}$ (Figure 34). Highest mortality occurred the first week after fish introduction in the tanks containing TEP from decaying *P. globosa* colonies (1.12 and 2.05 % $d^{-1}$).

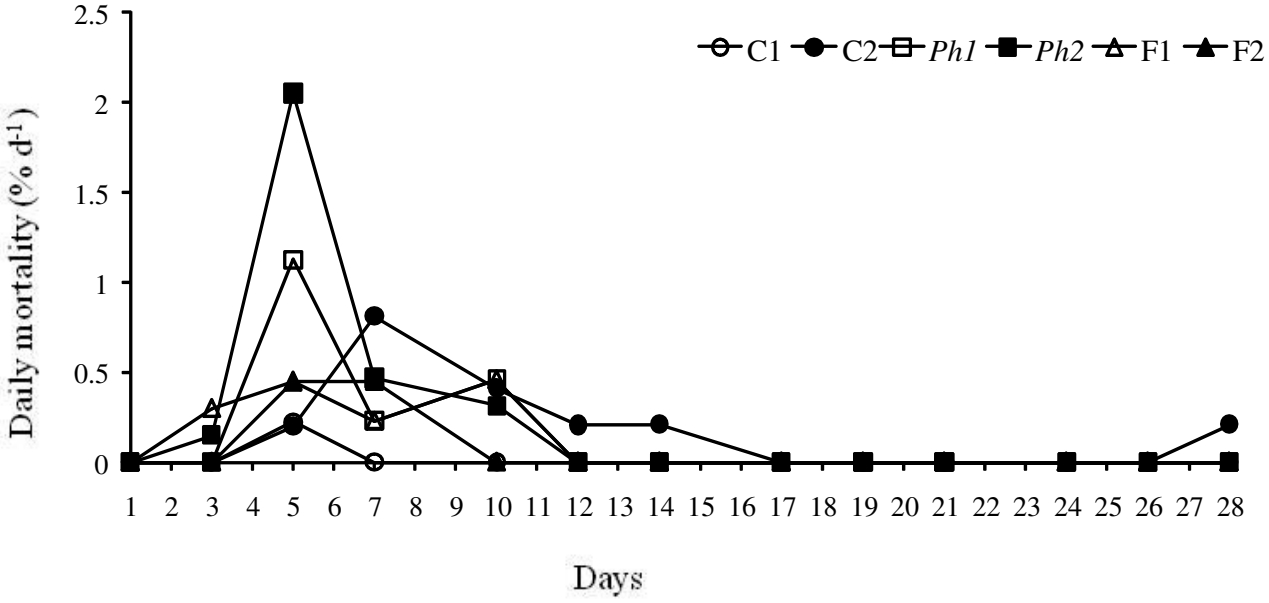



Figure 34. Evolution of the sea bass juvenile daily mortality rate (% $d^{-1}$) according to treatments: Controls (C1 and C2); *Phaeocystis* (*Ph1* and *Ph2*) and Foam (F1 and F2). Note that although all symbols are depicted on Figure 2 some of them are masked from day 10 to day 28

Growth of juvenile sea bass was generally similar both between replicates and treatments. No significant differences among replicates were recorded over the experiment except at $t_{28}$ where fish size between *Ph1* and *Ph2* were significantly different (KW, $p<0.0001$). The same holds for weight at $t_{28}$ between control treatments C1 and C2 (KW, $p<0.0001$).

For all sampling dates ($t_8$, $t_{20}$ and $t_{28}$), there was no significant difference in fish size or weight among conditions (KW, $p>0.05$) except at $t_{28}$ where fish size and weight were significantly higher in *Ph1* (42.01 ± 3.17 mm; 1456.69 ± 320.38 mg) and *Ph2* (43.42 ± 3.20

mm; 1581.59 ± 362.21 mg) compared to the Control (C1; 41.73 ± 2.99 mm; 1407.93 ± 326.47 mg) (KW, $p<0.0001$) (Table 13).

During the 28 days of the rearing experiment, the size of the juvenile sea bass increased from 34.81 ± 2.33 mm (SL) to 43.42 ± 3.20 mm and the weight from 773.62 ± 168.71 to 1581.59 ± 362.21 mg (Figure 35).

Juvenile sea bass specific growth rates in length ranged between 0.19 % $d^{-1}$ (*Ph1* at $t_8$) and 0.84 % $d^{-1}$ (F2 at $t_{20}$) whereas weight specific growth rate ranged between 0.98 % $d^{-1}$ (*Ph1* at $t_8$) and 3.23 % $d^{-1}$ (F2 at $t_{20}$; Table 13). Apart from the first rearing week, growth rates in length and weight were very close both between replicates and between conditions.

The Fulton's K condition index varied between 1.72 ± 0.15 and 2.11 ± 0.16 mg $mm^{-3}$ and significantly increased in all treatments throughout the experiment (Figure 35, Table 13). After one week ($t_8$), K was highly comparable to $t_0$ value (KW, $p<0.0001$) and significantly higher in the control (C1) compared to *Phaeocystis* and Foam conditions. This discrepancy was shaded over the course of the experiment and at $t_{20}$, no significant differences of K values were recorded between conditions (KW, $p>0.05$). By contrast, at the end of the experiment ($t_{28}$), K was significantly higher in the Foam treatments (F1 and F2) compared to control C1 whereas C2 exhibited significantly higher K values than the other treatments (KW, $p<0.0001$).

The RNA-DNA ratio varied between 2.40 ± 0.76 ($t_0$) and 3.12 ± 0.78 (*Ph1* at $t_{28}$; Figure 36, Table 13). At the end of the experiment ($t_{28}$), the RNA-DNA ratio in *Phaeocystis* and Foam conditions did not highlighted significant difference between replicates (KW, $p>0.05$). By contrast, individuals from the *Phaeocystis* conditions exhibited a significantly higher RNA-DNA ratio compared to Control and Foam conditions (KW, $p=0.002$; Figure 36). The observed increasing trends in RNA-DNA ratio between $t_0$ and $t_{28}$ are only significant for individuals reared under *Phaeocystis* conditions (KW, $p=0.009$; Figure 36; Table 13).

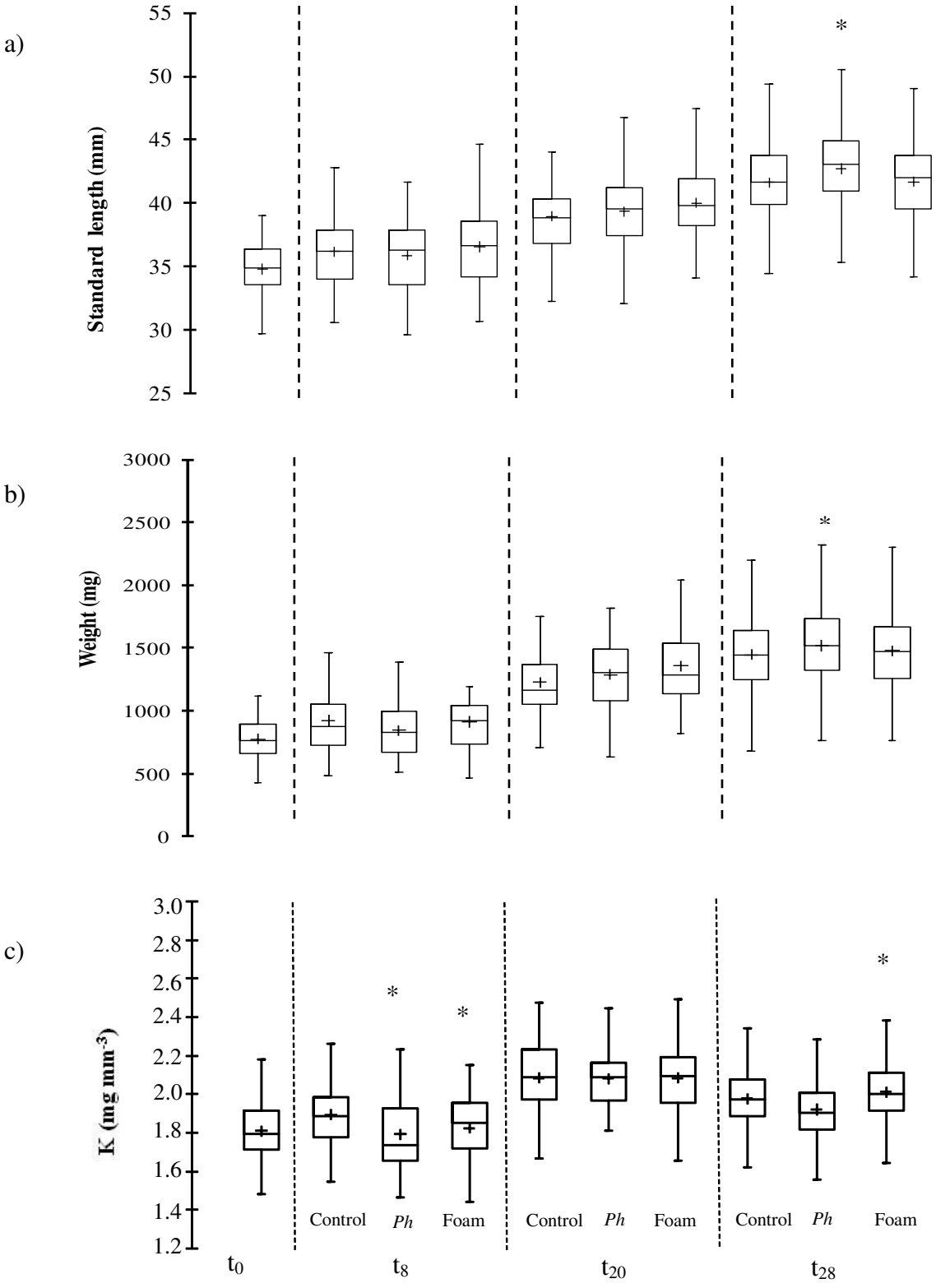



Figure 35. Box and whisker plots of sea bass juvenile size: standard length (a; mm), weight (b; mg) and Fulton's condition index *K* (c; mg $mm^{-3}$) in control, *Phaeocystis* and Foam treatments for each sampled week ($t_0$, $t_8$, $t_{20}$ and $t_{28}$). Whiskers extend to the highest and lowest values. Median (-) and arithmetic mean (+) are also indicated. * denotes a significant effect of treatment compared to control (KW, $p < 0.05$)

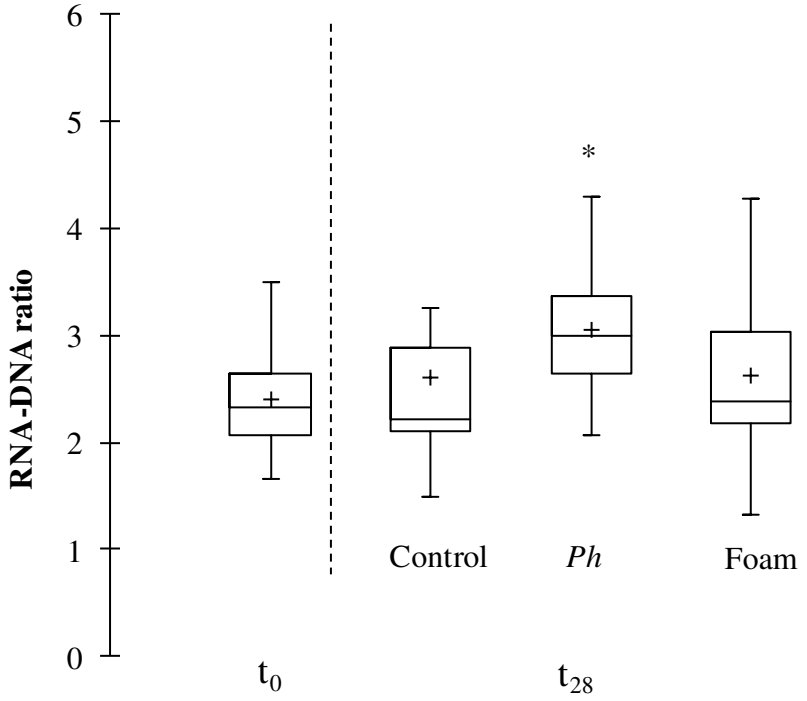



Figure 36. Box and whisker plots of sea bass juvenile RNA-DNA ratio at the beginning ($t_0$) and the end ($t_{28}$) of the mesocosm experiment for each condition (Control, *Phaeocystis* and Foam). * denotes a significant effect of treatment compared to control (KW, $p < 0.05$)

Table 13. Mean (± SD) values of biological parameters measured on sea bass juveniles exposed to TEP production. Depicted parameters are standard length (SL, mm), growth rate in length (GL; %.$d^{-1}$), body weight (mg), growth rate in weight (GW, %.$d^{-1}$), Fulton's condition index (K), and RNA-DNA ratio for each experimental treatment. Superscript ($^1$) and ($^2$) stands for significant difference ($p<0.05$) when compared to "$t_0$ and "control" respectively

| Dates (days) | N | Conditions | Standard length (SL, mm) | GL (% $d^{-1}$) | Body weight (W, mg) | GW (% $d^{-1}$) | K (mg $mm^{-3}$) | RNA-DNA ratio |
|---|---|---|---|---|---|---|---|---|
| $t_0$ | 50 | - | 34.81 ± 2.33 | | 773.62 ± 168.71 | | 1.81 ± 0.17 | 2.40 ± 0.76 |
| $t_8$ | 30 | C1 | 35.79 ± 2.90 | 0.43 | 901.19 ± 276.12 | 2.18 | 1.91 ± 0.17$^1$ | - |
| | 30 | C2 | 36.58 ± 3.01 | 0.73 | 941.26 ± 267.31 | 2.80 | 1.88 ± 0,15 | - |
| | 30 | *Ph*1 | 35.21 ± 2.80 | 0.19 | 828.63 ± 231.92 | 0.98 | 1.86 ± 0.23 | - |
| | 30 | *Ph*2 | 36.57 ± 2.61 | 0.73 | 854.44 ± 199.85 | 1.70 | 1.72 ± 0.15$^2$ | - |
| | 30 | F1 | 36.48 ± 3.07 | 0.70 | 878.70 ± 247.17 | 1.82 | 1.77 ± 0.15$^2$ | - |
| | 30 | F2 | 36.66 ± 2.86 | 0.77 | 944.92 ± 241.71 | 2.86 | 1.88 ± 0.17$^1$ | - |
| $t_{20}$ | 30 | C1 | 38.67 ± 2.22 | 0.56 | 1197.81 ± 234.16 | 2.30 | 2.05 ± 0.21$^1$ | - |
| | 30 | C2 | 39.23 ± 4.92 | 0.63 | 1249.97 ± 276.14 | 2.53 | 2.11 ± 0,37$^1$ | - |
| | 30 | *Ph*1 | 38.85 ± 3.48 | 0.58 | 1231.02 ± 279.46 | 2.45 | 2.05 ± 0.21$^1$ | - |
| | 30 | *Ph*2 | 39.83 ± 2.92 | 0.71 | 1341.81 ± 319.11 | 2.90 | 2.07 ± 0.13$^1$ | - |
| | 30 | F1 | 39.24 ± 2.52 | 0.64 | 1285.56 ± 251.83 | 2.67 | 2.09 ± 0.15$^1$ | - |
| | 30 | F2 | 40.80 ± 2.82 | 0.84 | 1429.48 ± 370.02 | 3.23 | 2.11 ± 0.16$^1$ | - |
| $t_{28}$ | 165 | C1 | 41.73 ± 2.99 | 0.65 | 1407.93 ± 326.47 | 2.14 | 1.90 ± 0.15$^1$ | 2.61 ± 0.99 |
| | 165 | C2 | 41.52 ± 2.78 | 0.63 | 1488.85 ± 296.46 | 2.34 | 2.06 ± 0,14$^1$ | - |
| | 165 | *Ph*1 | 42.01 ± 3.17 | 0.68 | 1456.69 ± 320.38 | 2.26 | 1.94 ± 0.16$^2$ | 3.12 ±0.78$^{1,2}$ |
| | 165 | *Ph*2 | 43.42 ± 3.20 | 0.79 | 1581.59 ± 362.21 | 2.55 | 1.91 ± 0.16$^2$ | 2.99 ± 0.45$^{1,2}$ |
| | 165 | F1 | 41.73 ± 2.85 | 0.65 | 1459.30 ± 309.79 | 2.27 | 1.98 ± 0.13$^{1,2}$ | 2.63 ± 0.76 |
| | 165 | F2 | 41.66 ± 3.11 | 0.64 | 1496.55 ± 322.13 | 2.36 | 2.04 ± 0.15$^{1,2}$ | 2.62 ± 0.88 |

IV.1.4. Discussion

Mesocosms are defined as experimental systems that simulate real-life conditions as closely as possible, whilst allowing the manipulation of environmental factors. Mesocosm experiments have played an important role over the last decade in increasing our understanding of marine ecosystems. Many studies used these controlled environments to examine ecosystem responses to factors such as nutrient addition and light limitation e.g. essentially factors driving phytoplankton (Li et al., 1992; Egge and Hemidal, 1994; Stephen et al., 2004) and the planktonic food web dynamics (Nowlin and Drenner, 2000; Romo et al., 2004). Mesocosm systems using macro-organisms as target species have mainly focused on exposure experiments to pollutants on either macrobenthos (Gee et al., 1985; Warwick et al., 1988) or fish (Tana et al., 1994; Bony et al., 2008). Few studies have considered mesocosm experiments under rearing conditions (Gyllenhamar et al., 2008; Deutsch et al., 2009) although such studies can provide new insights into fish farming management.

Our mesocosm experiment allowed the observation of juvenile survival and growth at near natural densities. As a result, responses of the juveniles to controlled alteration of their environment, such as high TEP concentrations mediated either from *Phaeocystis globosa* senescence or foam can be extrapolated to natural systems with much greater confidence than for studies done in smaller laboratory systems. Results from our experiments clearly allowed us to reject our initial hypothesis. In fact, *P. globosa* senescence and its mediated exudates had no negative effect on the mortality, growth performance and the general welfare of juvenile sea bass analysed under fish farming conditions.

TEP are formed from the coagulation of *Phaeocystis* exudates (acidic polysaccharides) and, in major part, from the release of large fragments of the mucilaginous colonial matrix (Alldredge et al., 1993; Passow and Alldredge, 1995a, b; Passow, 2002a, b; Mari et al., 2005). Margalef (1978) and Sournia (1982) early suggested that mucus sheaths as well as more dispersed polymers excreted by algae represent increased viscosity that may be used by phytoplankton to manage flow fields. Biologically induced modification of seawater viscosity following during phytoplankton bloom has also been acknowledged (Jenkinson, 1986, 1993; Ramus and Kenney, 1989; Jenkinson and Biddanda, 1995). Viscosity may affect number of biological processes such as predator–prey and sexual partner encounter rates (Gerritsen and Strickler, 1977; Kiørboe and Saiz, 1995), motility and swimming speed of microorganisms (Mitchell, 1991) and respiration and excretion in the gills of fishes (Jenkinson, 1989). It is

therefore likely that phytoplankton blooms and its inherent amounts of exudates (i.e. TEP) alter fish physiology and therefore, physiological performance.

In our study, significant differences in TEP concentrations of one order of magnitude were found between *Phaeocystis* (*Ph;* 7634.27± 3514.41 µg XG eq $L^{-1}$) and foam (*F;* 932.54 ± 341.21 µg XG eq $L^{-1}$) treatments. In both treatments TEP concentrations followed the same trend and increased throughout the experiment. This increase is congruent with frequent addition of foam and TEP within the tanks as well as with Mari et al. (2004) observation during the wane of the *P. globosa* bloom. Our TEP concentrations were high compared to natural concentrations (100 - 255 µg XG eq $L^{-1}$; Riesebell et al., 1995) although Radić et al. (2004) mentioned annual ranges of 4 to 14 800 µg XG eq $L^{-1}$ in the North Adriatic (Po River, Italy) during mucilage events associated to diatom blooms. Data from a two year survey (2007-2009) in the eastern English Channel indicated that TEP concentrations ranged between 393 and 4341 µg XG eq $L^{-1}$ during *P. globosa* blooms (Breton pers. com.). Klein et al. (2011) obtained maximum TEP concentrations of up to 1735 and 3604 µg XG eq $L^{-1}$ for sites of the eastern and western English Channel, exposed to river discharge and oceanic influence, respectively. These high TEP concentrations did not seem to negatively impact on juvenile fish. In fact, mortality was low and occurred mainly at the beginning of the experiment when TEP concentrations were low. In addition, no morphological abnormalities or lesions were observed in the tanks during the exposition to TEP produced by *P. globosa* decaying colonies or foam.

Over the experiment, hydrological conditions (temperature, salinity, dissolved oxygen, turbidity) were similar to those observed in the English Channel when juvenile sea bass use shallow coastal nursery grounds (Selleslagh and Amara, 2008a). In addition, measured physico-chemical parameters appeared to be favorable for juvenile sea bass growth. In fact, specific growth rates in length (GL; 0.19 to 0.84 % $d^{-1}$) and weight (GW; 0.98 to 3.23% $d^{-1}$) were within the range of published values for food replete fishes varying from 0.2 to 0.4 % $d^{-1}$ GL ( Millot et al., 2008) and from 2.2 to 4.55 % $d^{-1}$ GW (Hatziathanasiou et al., 2002; Valente et al., 2007).

During the 28 days of experiment, juvenile sea bass size increased by 7.2 mm (0.26 mm $d^{-1}$) and their weight by 708 mg (25.29 mg $d^{-1}$). Ré et al. (1986) obtained a growth rate of 0.24 mm $d^{-1}$, using otolith daily increment analysis, in captive juvenile sea bass. Our growth rates are congruent with those recorded for wild juvenile sea bass in Portuguese estuaries (0.48 - 0.51 mm $d^{-1}$; Vinagre et al., 2009a, b; Vasconcelos et al., 2009) and near Marseille

(France; 0.25 mm $d^{-1}$; Guérin-Ancey, 1973). In the eastern English Channel the growth rate was found to be about 0.32 mm $d^{-1}$ (Selleslagh et al., 2009). This suggests that mesocosms of $1m^3$ volume are sufficient to study juvenile fish at low density since they have negligible effects on both fish growth and mortality. Data from our experiments can therefore be considered robust and relevant as rates obtained from mesocosms are close to real-life conditions.

In all treatments, the condition factor K was much higher in our experiment (1.81 to 2.11 mg $mm^{-3}$) compared to wild juvenile sea bass (0.88 - 1.22 mg $mm^{-3}$; Vasconcelos et al., 2009; Fonseca et al., 2011). This suggests a better feeding of fish. Since only TEP concentrations varied between the treatments and throughout the experiment it is likely that these particles played a role in enhancing juvenile performance. In fact, significantly higher SL, weight and/or K index were recorded when TEP from decaying *P. globosa* colonies or Foam were added to mesocosms (Fig. 3 and Table 2).

Measures of fish growth and condition used in the present study are very sensitive to the fish environment and have thus been frequently used to assess habitat quality (*e.g*. Phelan et al., 2000; Fonseca et al., 2006; Amara et al., 2009). Nucleic acid quantification and subsequent RNA–DNA ratios have been used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Gwack & Tanaka, 2001). In all treatments, at day 28 (end of experiment), a length and weight gain was recorded, indicating that fishes globally adapted to their environment, and particularly, when TEP were provided as decaying *P. globosa* colonies (Fig. 4).

Fish feeding on detritus has already been reported, particularly for tropical fishes (Bowen, 1983; Adams 1976; Bowen and Allanson, 1982; Ahlgren, 1996; Wilson, 2002; German and Miles, 2010 and references therein). In temperate ecosystems, few fish are detritivorous and this feeding behaviour is often restricted to season and to members of the families Cyprinidae and Catostomidae (McNeely, 1987). For instance, the capacity of the juvenile white sucker *Catostomus commersoni* to selectively feed on 26-38 µm sized detritus was observed during laboratory experiments and field surveys (Ahlgren, 1996). In a more recent study, the diet of American brook lamprey larvae (*Lampetra appendix*) from Minnesota streams was shown to be dominated numerically by diatoms whereas organic detritus comprised the bulk (>85%) of ingested materials (Mundhal et al., 2005). Sea bass juveniles feeding on detritus or aggregates has not been reported so far although it is likely that they directly or indirectly benefited from the high TEP concentrations during our experiments.

TEP are sticky (Logan et al., 1995; Engel, 2000), gel-like particles of up to several hundred micrometers in size that exist as discrete particles or become attached to other particles (Alldredge et al., 1993; Passow and Alldredge, 1994). In fact, TEP generally serves as a matrix for aggregates with abundant particle intrusions of bacteria, phytoplankton and detritus in nature (Mari and Kiørboe, 1996; Passow and Alldredge, 1999). They can also adsorb dissolved organic matter such as trace metals (Santschi et al., 2006) and amino acids (Schweitzer et al., 2001). TEP chemical composition varies depending on the TEP precursors, i.e. polysaccharides released by various species, and on the prevailing growth conditions (Passow, 2002a), as well as on bacterial activity, since bacterial transformation, degradation and production of exudated organic matter may play important role in TEP dynamics (Ramaiah et al., 2000 and Passow, 2002a). These specific properties (flocculation, aggregation, adherence and composition) of TEP enhance marine snow formation (Simon et al., 2002) and improve their nutritional values being thus direct non motile and suitable sized food source for sea bass juveniles. Fish feeding on marine snow has already being reported at laboratory for juvenile white and stripped mullets (Larson and Shanks, 1996). Although exclusive feeding on marine snow did not trigger positive growth, assimilation efficiency of organic matter and total amino acid from marine snow averaged 50%.

The Sea bass *Dicentrarchus labrax* can adapt to different feeding strategies and are generally voracious. It is a species of high commercial value in Europe and the quality of the nursery areas that sustain the juveniles may influence the viability of the coastal stocks (Beck et al., 2001). Adults migrate to coastal areas during the spawning season. Larvae and juveniles develop in the coast and then migrate to sheltered coastal areas or estuarine nurseries where they spend their first years of life (Amara and Paul, 2003; Selleslagh et al., 2009). The results obtained in the present study clearly showed that exudates and TEP excreted from decaying *P. globosa* colonies and foam accumulation have no negative effect on juvenile sea bass. Surprisingly, high TEP concentrations derived from *P. globosa* decaying colonies and foam had rather a positive impact on juvenile sea bass improving their physiological performance, hence, their survival and recruitment success. These results are congruent with the study carried out *in situ* by Selleslagh and Amara (2008a). These authors found that despite considerable interannual variability in the *P. globosa* spring bloom magnitude (Factor 40), no effect was observed on both fish and macrocrustacean species densities and diversity. Although direct or indirect benefits from TEP were suggested, further experiments (e.g. targeted feeding experiments) are needed to assess whether they contribute to the diet of sea bass juveniles.

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## CHAPTER IV - 2

# DOES *Pseudo-nitzschia pseudodelicatissima* CAN BE DELETERIOUS TO THE GROWTH AND CONDITION OF EUROPEAN SEA BASS JUVENILES?

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**ABSTRACT**

The aim of this study is to analyse the effect *Pseudo-nitzschia pseudodelicatissima* blooms in two physiological phase (exponential versus senescent state) on the physiological performance and mortality of European sea bass juveniles. Thus, during a 21 days mesocosm experiment (1 $m^3$ enclosures), sea bass juveniles (103 days old) were exposed to high *P. pseudodelicatissima* concentrations (1 – 9E + 06 cell $L^{-1}$ in exponential phase and 1 – 6E + 06 cell $L^{-1}$ in senescent phase). Environmental parameters (pH, dissolved oxygen, temperature, and salinity) were stable, similar among treatments and not limiting for growth. Fish mortality rate was relatively low (0 to 0.13 % $d^{-1}$) and occurred mostly during the first week when *P. pseudodelicatissima* concentrations were low. Biological performances (growth in length and weight, Fulton condition index and RNA-DNA ratio) of juvenile sea bass were generally similar both between replicates and conditions, unless TAG-ST ratio which showed significant decrease in senescent phase compared to control tank at $t_{21}$. During the 21 days of experiment, juvenile sea bass size increased by 8.5 mm (0.41 mm $d^{-1}$) and the weight by 956 mg (45.54 mg $d^{-1}$). The results obtained clearly showed that *Pseudo-nitzschia pseudodelicatissima* blooms have no negative effect on juvenile sea bass physiological performance and survival and hence on their recruitment success.

**Keywords:** *Dicentrarhus labrax*, algal bloom, *Pseudo-nitzschia pseudodelicatissima*, physiological performance indices, mesocosm

## IV.2.1 Introduction

Estuaries and their associated shallow coastal marine habitats provide spawning and nurseries grounds and present a high level of productivity for many marine fishes, including commercial fish species (Beck et al., 2001). However, these areas are often vulnerable to various stressors, both natural and anthropogenic, which may modify their ecological functions (Meng et al., 2001; Amara et al., 2007). Indeed, the ecological function of coastal areas around the world may be disturbed by a variety of environmental problems, including loss or destruction of habitats and decline of coastal waters quality. Among the stressors affecting the ecology of coastal areas, High Biomass Harmful Algal Blooms (HB-HABs) are perceived to be an increasing environmental, health and economic problem worldwide. These areas have been experienced with great diversity and abundance of HB-HABs and its toxic events (e.g. due to the climate change, increased ocean eutrophication and commercial shipping) over the last several decades (Anderson et al., 2002).

HB-HABs composed of many various phytoplankton species capable of producing biotoxins and may cause serious harm through the production of their toxins or by their accumulated biomass. Thus, this natural phenomenon can alter food web dynamics and cause substantial economic losses to coastal communities and commercial fisheries, as well as harmful algal bloom-associated fish, shellfish, bird and mammal mortalities. Some algal toxins are extremely strong, and low density blooms can be dangerous, sometimes causing poisonings at concentrations as low as a few hundred cell $L^{-1}$. Beside this, the monitoring of marine toxins is vital to the aquaculture industry, as these toxins may cause substantial ecological damage and economic losses through frequent or prolonged contamination and closure of harvesting sites (Hoagland et al., 2006; Falconer, 2008; Stewart et al., 2008; Campbell et al., 2010). For this reason, many effective research and monitoring programs have been established to monitor and assess HB-HABs risks in marine organisms under the control of some regulatory agencies along the coastal waters on the US West Coast, Canada, Norway, Japan, Australia, New Zealand and in some European countries such as France, Scotland, Ireland, Portugal, Spain (Lefebvre and Robertson, 2010).

*Pseudo-nitzschia spp.* (Bacillariophyceae) is one of the most recurrent phytoplankton blooms recorded in the northwest European shelf seas. *Pseudo-nitzschia* blooms are common in marine and estuarine environment and have deep socio-economic impacts on shellfish farming or harvesting and fishermen, but were recognized as being potentially toxic only 20 years ago (Bates et al. 1989; Klein et al., 2010). The genus is a pennate small needle-shaped

marine diatom which are not mobile in the water column (unlike dinoflagellates), and are therefore highly dependent on nutrient intakes and can be recognized by its characteristic "step-chain'' formation. They are frequently observed in North America: California, Washington, Bay of Fundy, Prince Edward Island, British Colombia (Hallegraeff, 2003) and more recently in Europe: Western Spain (Miguez et al., 1996), Western Scotland (Campbell et al., 2001; Gallacher et al., 2001; Fehling et al., 2004), Ireland (Cusack et al., 2002), and France (Nezan et al., 2006). In addition, *P. pseudodelicatissima* belonging to this genus is a spindle-shaped diatom species, living in short but lasting colonies found in tropical and temperate coastal waters and are abundant in the spring and summer. This species (usually as single or formed pairs) colonized also the surface of the *Phaeocystis globosa* colonies at all depths from the Eastern English Channel (Fryxell et al., 1997; Kaczmarska et al., 2005; Sazhin et al., 2007). Considerable research has been conducted in an effort to understand diversity, distribution, abundance, population dynamics, accumulation, grazing and growth, neurotoxicity, genotoxicity and effects of environmental factors of this toxic genus (Scholin et al., 1994a, 1994b; Miller and Scholin, 1996; Pan et al., 1996a, 1996b; Trainer et al., 2000, 2002; Fehling et al., 2004, 2005; Kudela et al., 2005; Kaczmarska et al., 2005, 2007; Thessen and Stoecker 2008 ; Olson et al., 2008, Olson and Lessard 2010; Stewart, 2010). Members of the genus *Pseudo-nitzschia* have been confirmed as producers of the excitatory neurotoxin, domoic acid (DA), causing amnesic shellfish poisoning (ASP) in humans and domoic acid poisoning (DAP) in marine organisms, mammals and seabirds (Thessen and Stoecker, 2008).

*Pseudo-nitzschia* blooms have been observed across the French coast, especially in spring (from April to June), by REPHY (Phytoplankton and Phycotoxins Monitoring Network), with maximum annual key concentrations, often exceeding 100.000 cells $L^{-1}$ and frequently more than 1 million cells $L^{-1}$. The toxic species, *P. pseudodelicatissima* had been detected in different regions of France in recent years, but at low concentrations. A first assessment in France in 1995 showed no trace of DA in shellfish during blooms of *Pseudo-nitzschia spp* (Le Doux et al., 1996). However, traces were detected in 1998 in mussels (0.5 mg DA $g^{-1}$ meat) from Côtes d'Armor (English Channel) related to the presence of *P. pseudodelicatissima*. Since 1999, DA assays in shellfish have been performed systematically in France by REPHY in accordance with recent EU regulatory decisions, i.e. as soon as the number of *Pseudo-nitzschia spp*. rises above $10^5$ cells $L^{-1}$ (the mean threshold for Europe) (Amzil et al., 2001). REPHY detected also DA above the EU-regulatory limit of 20 mg DA $g^{-1}$ wet weight of tissue in king scallops (*Pecten maximus*) from the Bay of Seine. In May 2000, one of the most important developments of this species has led to the presence of

amnesic shellfish poisoning (ASP) in shellfish in the Iroise Sea and the Bay of Douarnenez (West Brittany, France). In November 2004, Nezan et al. (2006) found a few empty frustules of *Pseudo-nitzschia* species in sediment of many affected sites in the Bay of Seine whereas neither in bottom waters nor at the surface, *Pseudo-nitzschia* species was observed (Klein et al., 2010).

There are many way of DA accumulation. This neurotoxin may enter the food chain from diatoms via filter-feeding shellfish or finfish. The toxin then accumulates to such levels that ingestion of the vectors by humans or other animals may lead to sickness or mortality in sea mammals, seabirds and humans due to ASP. Numerous laboratory-based toxicity studies were performed in order to characterize the neurotoxicity of DA has been observed in several animal species including humans, non-human primates, rodents, rats, fish, marine mammals and birds (Iverson et al., 1990; Tryphonas et al., 1990; Tasker et al., 1991 ; Lefebvre et al., 2001 ; Schaffer et al., 2006). Its neurotoxic effects in adult animals have been the subjects of several studies and are summarized in various excellent reviews (Todd, 1993; Mos, 2001; Pulido, 2008; Lefebvre and Robertson, 2010). To date, these harmful algae have become a focal point of numerous ecological studies and monitoring efforts in recent years and are subjected of various aspects of DA toxicology, pathology, bioaccumulation, and production in toxic diatom species. (Fehling et al., 2005**;** Schnetzer et al., 2007**;** Costa et al., 2010)**.** Nevertheless, the reason for domoic acid production is not fully understood. Laboratory analyses show cultures of *Pseudo-nitzschia* produce domoic acid under silicate or phosphate limitation, but not nitrogen or light limitation. Field studies in the Pacific Ocean and laboratory studies have found increased domoic acid production under conditions of iron limitation. Many other studies were done about DA production under nutrient stress by several researchers (Pan et al., 1996a; Pan et al., 1998; Bates, 1998; Bates et al., 2000; Maldonado et al., 2002; Wells et al., 2005; Trainer et al., 2009).

The possible toxic effects of *Pseudo-nitzschia pseudodelicatissima* on the growth and condition indices of aquatic organisms, especially of fish juveniles, are scarcely investigated (Dizer et al., 2001; Tiedeken et al., 2005; Lefebvre et al., 2007). Thus the goal of this study was to investigate the effects of two physiological phases (exponential versus senescent state) of *Pseudo-nitzschia pseudodelicatissima* on the physiological performances of sea bass juveniles (*Dicentrarchus labrax*). This species is easy to handle and maintain in rearing conditions and their juveniles, as many other marine species, use shallow marine coastal areas as nursery grounds, areas where *P. pseudodelicatissima* bloom generally occur. To date, it is

the first study devoted to evaluation of the physiological conditions in early life stage of fish exposed to this algae species in mesocosm.

## IV.2.2. Materials and Methods

### IV.2.2.1. *Pseudo-nitzschia pseudodelicatissima* algal cultures

*P. pseudodelicatissima* strain was isolated from the eastern English Channel in spring 2009 pippeting cells from coastal water samples. *P. pseudodelicatissima* was maintained in monospecific conditions in f/2 medium (Guillard and Ryther 1962; Guillard, 1975) at 12 ± 0.5°C with a 12:12 h light:dark cycle under a photon flux density of 400 µmol photons $m^{-2}$ $s^{-1}$ (Daylight HQIT-WD 250 W F, OSRAM). Prior to the experiment, this species was grown for 1 and 2 weeks under the same light conditions in a 250L and 100L plexiglass batches filled with UV sterilized seawater at 15.0 ± 0.5°C. First batch (250 L) was served to maintain exponential phase for *Ps1* and *Ps2* tanks where the initial concentration was ~$10^8$ cell $L^{-1}$ (e.g. >$10^7$ cell $L^{-1}$; Hasle 2002). In batch cultures, it is assumed that DA production often starts at the onset of stationary phase of *P. pseudodelicatissima* and peaks about one week later (Pan et al., 2001). For this reason, second batch (100 L) was served as senescent phase of *P. pseudodelicatissima* for *Ps3* tank, where domoic acid production can be produced.

### IV.2.2.2. Experimental set up and sampling strategy

In order to explore the effects of *P. pseudodelicatissima* physiological states derived material on fish survival and physiological performances, a first mesocosm experiment was carried at Aquanord hatchery over 21 days in 2009 (from 10 to 31 July), and a second with *P. pseudodelicatissima* in senescent phase which started one week later (from 17 to 31 July).

Five circular tanks of $1m^3$ each were supplied with sand-filtered and UV sterilized running seawater, thus permitting to keep mesocosm at *in situ* temperature (15 ±1°C) throughout the experiment. Tanks were illuminated according to the natural photoperiod at a photon density of 400 µmol $m^{-2}$ $s^{-1}$ (Daylight HQIT-WD 250 WF) and gently aerated (compressed air), thus preventing settling of particles and maintaining oxygen saturation above 80%.

Juvenile sea bass (1300 individuals) were obtained from the Aquanord hatchery and reared at low density (0.25 ind $L^{-1}$). At the beginning of the experiment ($t_0$), 50 individuals were randomly sampled, anesthetized for 3-4 minutes with 2-phenoxyethanol (0.32 mL $L^{-1}$ final concentration) and frozen (-20°C) for analyses of size, weight and condition.

250 individuals (aged 103 days old) were randomly distributed in the five tanks. Every 2 days, two tanks in exponential phase (*Ps1* and *Ps2*) and one thank (*Ps3)* in senescent phase received 50 L of *P. pseudodelicatissima* culture to maintain high and stable concentration in each tank. The last two tanks (C1 and C2) only contained juvenile sea bass and were used as controls. In each tank, 25% of the seawater was renewed every two days.

Fish were fed daily *ad libitum* every morning with commercial dry pellets (Skretting Ltd., France, Gemma PG 1.0) which contain 56% protein, 18% oil, 10% dry ashes, 0.6% fibre, 1.3% total phosphorus, copper (8 ppm $CuSO_4$), vitamin A (15000 I.U. / kg), vitamin D3 (1125 I.U. / kg) and vitamin E (225 I.U. / kg).

Daily observations were carried out every morning before the first food supply to assess fish mortality. Sampling and measurements were always done at a 2 days frequency before seawater renewal and fish feeding. Dissolved oxygen concentration (DO, mg $L^{-1}$), seawater temperature (°C), salinity, pH using a Hanna HI 9828 multiprobe and turbidity (NTU) using a turbidimeter (Eutech instruments, TN-100) were measured. For fish growth and physiological performances monitoring, 30 individuals from each tank were removed on day 8 and day 15. At the end of the experiment (day 21), all the remaining fishes were removed from the tanks. After each removal, fishes were anaesthetised and frozen at -20 °C.

#### IV.2.2.3. Sampling and determination of *Pseudo-nitzschia pseudodelicatissima* total abundances

A 40ml seawater sample was collected every two days before renewing seawater into the each tank to determine phytoplankton cell abundance. Samples were preserved with Lugol-gluteraldehyde (1% final concentration) and stored at 4°C in the dark until analysis (within one month). Total abundance of *P. pseudodelicatissima* (cell $L^{-1}$) was determined by inverted light microscopy according to the Utermohl method (Uthermohl, 1958).

IV.2.2.4. Fish mortality and physiological performance

Dead fishes were counted daily and instantaneous daily mortality coefficients (M, $d^{-1}$) were estimated for each condition and tank applying the exponential model of decline:

$$Nt = N_0 \exp^{-M\,(t-t0)}$$

where $N_t$ is the number of fishes at time $t$. $N_0$ is the number of fishes at the beginning of the experiment ($t_0$) , $(t-t_0)$ is the experiment duration (days), and M is the instantaneous daily mortality coefficient.

At the laboratory, morphometric measurements were made on a total of 1300 juveniles (50 for $t_0$, 30 per tank for $t_8$, $t_{15}$ and 190 per tank for $t_{21}$). Each juvenile was thawed at room temperature, weighed (W, fresh weight; mg) to the nearest 0.1 mg and measured for total length (TL, mm) to the nearest 0.5 mm.

Juvenile sea bass specific growth rates in weight (mg $d^{-1}$) were estimated as:

$$GW = 100(lnW_t - lnW_0)/(t-t_0),$$

where $W_0$ and $W_t$ are fish total body weight at times $t_0$ and $t$ (time of collection), respectively. Similarly, the specific growth rate in length (mm $d^{-1}$) was estimated as:

$$GL = 100(\ln L_t - lnL_0)/(t-t_0),$$

where $L_0$ and $L_t$ are fish standard length at times $t_0$ and $t$, respectively.

Three condition indices were estimated during the experiment: 1) RNA-DNA ratio and 2) TAG-ST ratio as indicators of nutritional status, 3) Fulton's K condition index as an indicator of the fish general well being. This latter morphometric index assumes that heavier fish for a given length are in better condition. Individual condition factor (K) was determined from morphometric data, according to the formula:

$$K = (100 \times W) / SL^3$$

where W is the body weight (mg) and TL is the standard length (mm).

The RNA-DNA and TAG:ST ratios were calculated at time $t_0$ and $t_{21}$ for each incubation condition (25 individuals for $t_0$ and 15 individuals per tank for $t_{21}$).

Nucleic acid quantification and subsequent RNA–DNA ratios have been used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Gwack and Tanaka, 2001). This biochemical index reflects variations in growth-related protein synthesis, since the quantity of ribonucleic acid (RNA) varies with the rate of protein synthesis, while the amount of deoxyribonucleic acid (DNA) per cell is species-constant in somatic tissue (Buckley and Bullow, 1987). The procedure used to determine RNA and DNA concentrations in individual fish is based on the Clemmesen

method (1988) slightly modified by Amara et al. (2009). However, heads and fins were discarded before analysing fish and guts were excised to ensure that gut contents did not contribute to RNA–DNA ratio. Fish muscle sample (0.05 g) was homogenized in ice−cold Tris–EDTA buffer (0.05 M Tris. 0.1 M NaCl. 0.01 M EDTA. pH 8.0) using an Ultraturrax and subsequently transferred to a mixture of Tris–EDTA buffer, proteinase−K (pro−K) and sodium dodecyl sulfate (SDS). Nucleic acids were extracted by purification steps involving phenol−chloroformisoamylalcohol (Amara et al., 2009). The quantity of RNA and DNA was determined by the fluorescence−photometric technique using a specific nucleic acid fluorescent dye−ethidium bromide (Sigma−Aldrich Chemicals. France). The fluorescence due to total RNA was calculated as the difference between total fluorescence (RNA and DNA) and the fluorescence after RNAase treatment which is assumedly to be due to the presence of only DNA. Salmon sperm DNA (Sigma−Aldrich Chemicals, F rance) and yeast type III RNA (Sigma−Aldrich Chemicals. France) were used as standards. RNA and DNA contents are both expressed as µg per µL.

The third condition index was a lipid storage index based on the ratio of the quantity of triacylglycerols (TAG; reserve lipids) to the quantity of sterols (ST; structural lipids) in the fish. The TAG content is dependent on the nutritional state of the fish as they are a main reserve of energy in teleosts and the first components to be mobilised during periods of stress, while sterol contents remain essentially unchanged during starvation (Amara et al., 2007). The amount of total lipids in each individual was measured on a sample of lyophilised muscle (0.07 g). Lipid extraction was conducted using the method of Bligh and Dyer (1959) slightly modified as described by Amara et al. (2007). Lipids were extracted according to a mixture of water:choloroform:methanol (1:1:1; v/v/v).TAGs and sterols were separated from other lipids by performing thin layer chromatography (TLC).

### IV.3.2.5. Statistical analysis

Since physico-chemical and biological data considered here did not comply with the parametric assumption of normality and variance equality, non−parametric Kruskall−Wallis test followed by the post hoc Dunn test (joint ranking test) were used for pair wise comparisons. *P. pseudodelicatissima* concentrations in the different conditions (*Ps1, Ps2* and *Ps3*) were analysed by Mann-Whitney test. A significance level of a minimum of 5 % was considered in all statistical analyses. The statistics were performed using XLSTAT software package (version 5.01).

## IV.2.3. Results

### IV.2.3.1. Physico-chemical variables

Physico-chemical parameters were stable over the 21 days of experiment, unless temperature, dissolved oxygen and turbidity (Table 14). Water temperature showed similar trends over the study, increasing the first two days from 14°C to 16°C and then stabilizing to 16-17°C until the end of the experiment. Salinity was also stable during the experiment and varied from 34.7 ± 0.7 to 34.9 ± 0.4 between treatments. Dissolved oxygen values showed variations the first three day between 25-36 mg $l^{-1}$ and then stabilized with 10-15 mg $l^{-1}$. pH conditions doesn't show any evident variations and ranged from 7.5 ± 0.3 to 7.7 ± 0.3 among conditions. Turbidity varied from 0.3 to 0.9 NTU the first three days and then stabilized to 1.0-1.5 NTU until the end of the experiment. No significant differences were observed for each parameter among the treatments (KW, $p>0.05$).

### IV.2.3.2. *Pseudo-nitzschia pseudodelicatissima* total abundance

Our results showed significant differences between culture of 250 L and culture of 100 L (Mann-Whitney, $p=0.001$) (Figure 37). Total abundance in culture of 250 L ranged from 1.01E + 08 to 6.30E + 08 cell $L^{-1}$. It was observed a peak on the abundance every week of this culture and showed slightly fluctuations nonetheless maintaining sufficient *P. pseudodelicatissima* abundances during the experiment. Total abundances in culture of 100 L ranged from 1.60E + 07 to 1.80E + 08 cell $L^{-1}$ and decreased gradually during the experiment as expected in senescent phase that can produce domoic acid.

Over the experiment, *P. pseudodelicatissima* total abundances originating from the culture of 250 L and 100 L, respectively, decreased by a factor of 100 in *Ps1* and *Ps2* and by a factor of 10 in *Ps3*. However, these values stayed at higher those as observed during *P. pseudodelicatissima* blooms in the Eastern English Channel ($10^3$ cell $L^{-1}$, Klein et al., 2010). Concentrations ranged from 1.00E + 06 to 8.00E + 06 cell $L^{-1}$ and from 1.00E + 06 to 9.00E + 06 cell $L^{-1}$, for *Ps1* and *Ps2*, respectively. In both tanks, the values increased softly at the beginning and reached the highest level at the last week of experiment. Mean total abundances were not significantly different between tanks reaching 2.50E + 06 cell $L^{-1}$ in *Ps1* and 2.91E + 06 cell $L^{-1}$ in *Ps2* (M-W, $p=0.828$). Concentrations ranged from 1.00E + 06 to

6.00E + 06 cell $L^{-1}$ in *Ps3* basin (Figure 38). However, there is a decrease of concentration in *Ps3* tank the second week of experiment.

Table 14. Environmental context of the mesocosm experiment. Mean values (± SD) of the physico-chemical parameters measured (temperature, salinity, dissolved oxygen, pH and turbidity) over the course of the experiment (10 to 31 July) in Control and *Pseudo-nitzschia pseudodelicatissima* treatments. Values in parentheses stand for the range ($X_{min}$-$X_{max}$) of each parameter

| Treatment | Temperature (°C) | Salinity | Dissolved oxygen (mg $L^{-1}$) | pH | Turbidity (NTU) |
|---|---|---|---|---|---|
| C1 | 16.3 ± 0.7 (14.4 – 16.8) | 34.7 ± 0.7 (34.0 – 36.4) | 13.6 ± 4.6 (9.4 – 25.0) | 7.6 ± 0.3 (7.2 – 8.3) | 1.0 ± 0.3 (0.6 – 1.5) |
| C2 | 16.5 ± 0.5 (14.4 – 16.9) | 34.8 ± 0.2 (34.3 – 35.2) | 12.2 ± 7.7 (8.4 – 33.7) | 7.6 ± 0.6 (7.1 – 8.7) | 0.9 ± 0.4 (0.3 – 1.5) |
| *Ps1* | 16.3 ± 0.7 (14.4 – 16.8) | 34.9 ± 0.4 (34.4 – 35.8) | 13.4 ± 6.5 (8.6 – 31.0) | 7.7 ± 0.3 (7.3 – 8.1) | 1.0 ± 0.4 (0.6 – 1.7) |
| *Ps2* | 16.5 ± 0.5 (14.4 – 17.0) | 34.9 ± 0.2 (34.5 – 35.4) | 13.7 ± 8.3 (9.4 – 36.8) | 7.7 ± 0.3 (7.3 – 8.2) | 0.8 ± 0.3 (0.3 – 1.5) |
| *Ps3* | 16.7 ± 0.2 (16.5 – 16.8) | 34.8 ± 0.1 (34.7 – 35.0) | 10.1 ± 1.2 (8.1 – 11.1) | 7.5 ± 0.3 (7.2 – 7.8) | 0.9 ± 0.3 (0.6 – 1.2) |

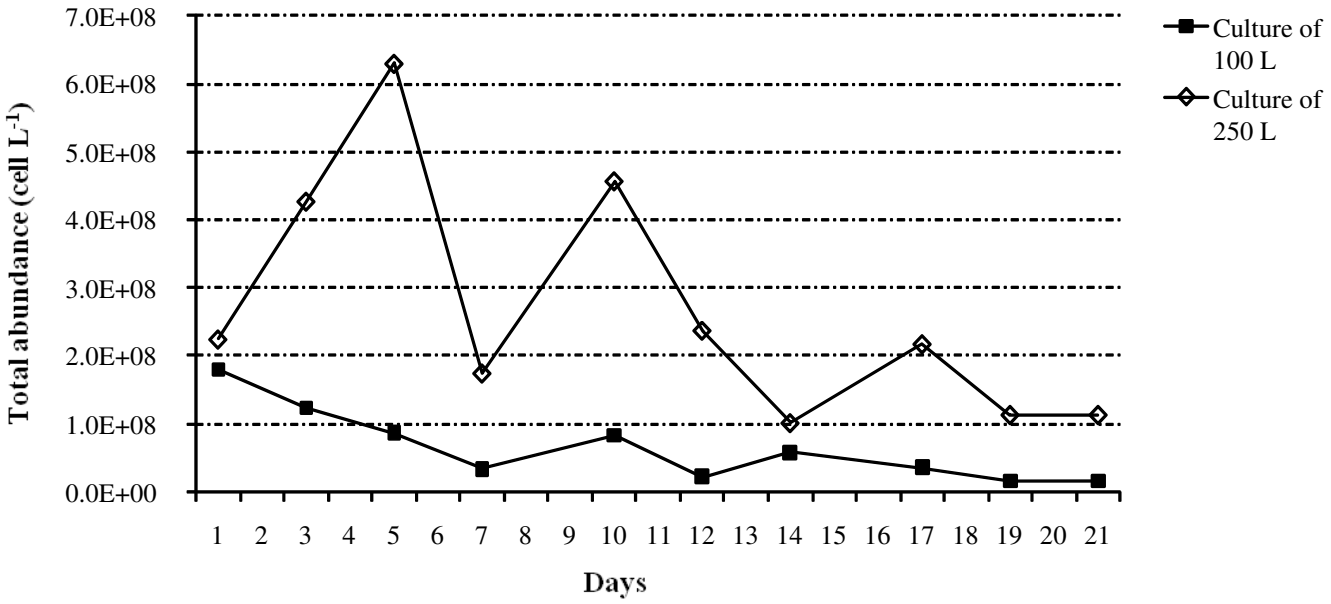


Figure 37. Abundance of *Pseudo-nitzschia pseudodelicatissima* in 250 L and 100 L batch cultures (cell $L^{-1}$) during experiment

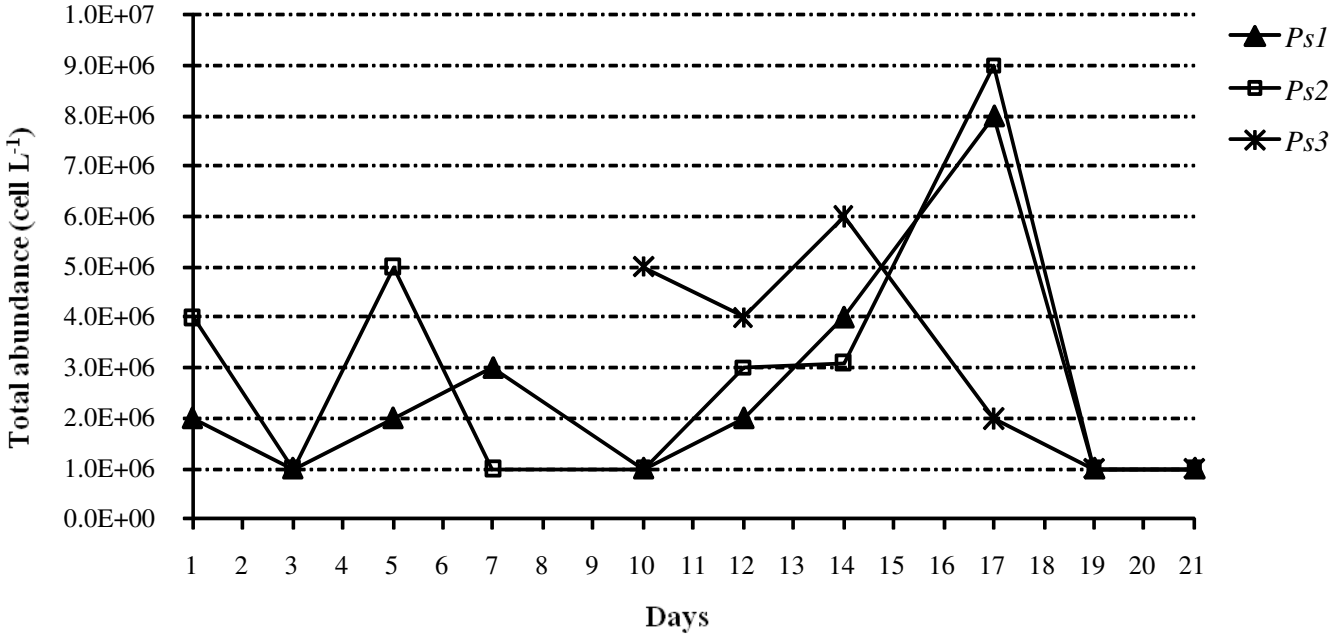


Figure 38. *Pseudo-nitzschia pseudodelicatissima* abundance in exponential phase (*Ps1* and *Ps2*) and senescent phase (*Ps3*) (cell $L^{-1}$) during experiment

IV.2.3.3. Fish mortality and physiological performance

No fish malformation, morphological abnormalities or lesions indicating cannibalism were observed during the course of the experiment. Mortality rate ranged from 0 to 0.13 % $d^{-1}$during the experiment. Only, highest mortality occurred the third day and the second week in the *Ps2* and C2 tanks, respectively (0.13% $d^{-1}$).

Growth of juvenile sea bass was generally similar both between replicates and treatments. No significant differences among replicates were recorded over the experiment except at $t_{21}$ where fish size between *Ps1* and *Ps2* were significantly different (KW, $p<0.0001$). The same holds for weight at $t_{21}$ between control (C1 and C2) and *Pseudo-nitzschia* (*Ps1* and *Ps2*) treatments (KW, $p<0.0001$).

For all sampling dates ($t_8$, $t_{15}$ and $t_{21}$), there was no significant difference in fish size or weight among conditions (KW, $p>0.05$) except at $t_{21}$ where fish size were significantly different in *Ps2* (57.30 ± 4.27 mm) compared to the control conditions (C1; 58.94 ± 4.45 mm; C2; 58.81 ± 4.24). This discrepancy observed also for fish weight at the end of experiment. *Ps1* (2527.93 ± 567.16) and *Ps3* (2608.42 ± 653.16) were significantly higher than C2 (2278.40 ± 511.51) whereas *Ps2* (2202.84 ± 514.21) exhibited lower significant difference compared to C1 (2498.61 ± 604.83) (KW, $p<0.0001$) (Table 15).

During the 21 days of the rearing experiment, the size of the juvenile sea bass increased from 50.17 ± 3.54 mm (TL) to 59.83 ± 4.49 mm and the weight from 1466.96 ± 368.77 to 2608.42 ± 653.16 mg (Figure 39).

Juvenile sea bass specific growth rates in length ranged between 0.34 % $d^{-1}$ (C2 at $t_8$) and 0.84 % $d^{-1}$ (*Ps3* at $t_{21}$) whereas weight specific growth rate ranged between 0.43 % $d^{-1}$ (C1 at $t_8$) and 2.38 % $d^{-1}$ (*Ps3* at $t_{21}$; Table 15). Apart from the first rearing week, growth rates in length and weight were very close both between replicates and between conditions.

The Fulton's K condition index varied between 1.10 ± 0.08 and 1.23 ± 0.10 mg $mm^{-3}$ and significantly increased in all treatments throughout the experiment (Figure 39, Table 15). After one week ($t_8$), K was highly comparable to $t_0$ value (KW, $p<0.0001$) and significantly higher in *Ps2* compared to control (C1) condition. The second week of the experiment ($t_{15}$), K was significantly higher in the control treatments (C1 and C2) compared to *Ps1*. At the end of the experiment ($t_{21}$) significant differences of K values were recorded between *Ps1, Ps2 and Ps3* compared to control conditions (C1 and C2) (KW, $p<0.0001$).

The RNA-DNA ratio varied between 1.74 ± 0.54 and 2.64 ± 1.46 (Figure 40a, Table 15). At the end of the experiment ($t_{21}$), the RNA-DNA ratio in *Pseudo-nitzschia* and control

conditions highlighted significant difference between replicates (KW, $p<0.001$). The RNA-DNA ratio of individuals from *Ps1* and *Ps3* were significantly higher compared to C2 whereas *Ps2* exhibited lower RNA-DNA ratio than C1 (KW, $p=0.002$; Table 15). The observed evident variations trends in RNA-DNA ratio between $t_0$ and $t_{21}$ did not show any significant difference for individuals reared under *Pseudo-nitzschia* conditions (KW, $p=0.093$; Figure 40a).

The TAG-ST ratio ranged from $0.47 \pm 0.48$ and $1.02 \pm 0.87$ (Figure 40b, Table 15). At the end of the experiment ($t_{21}$), the TAG-ST ratio in *Pseudo-nitzschia* and control conditions did not highlighted significant difference between replicates (KW, $p>0.05$). By contrast, individuals from *Ps3* exhibited a significantly lower TAG-ST ratio compared to control (C1) condition (KW, $p=0.010$; Table 15). The observed decreasing trends in TAG-ST ratio between $t_0$ and $t_{21}$ are only significant for individuals reared under *Pseudo-nitzschia* in senescent phase (*Ps3*) condition compared to control (KW, $p=0.004$; Figure 40b).

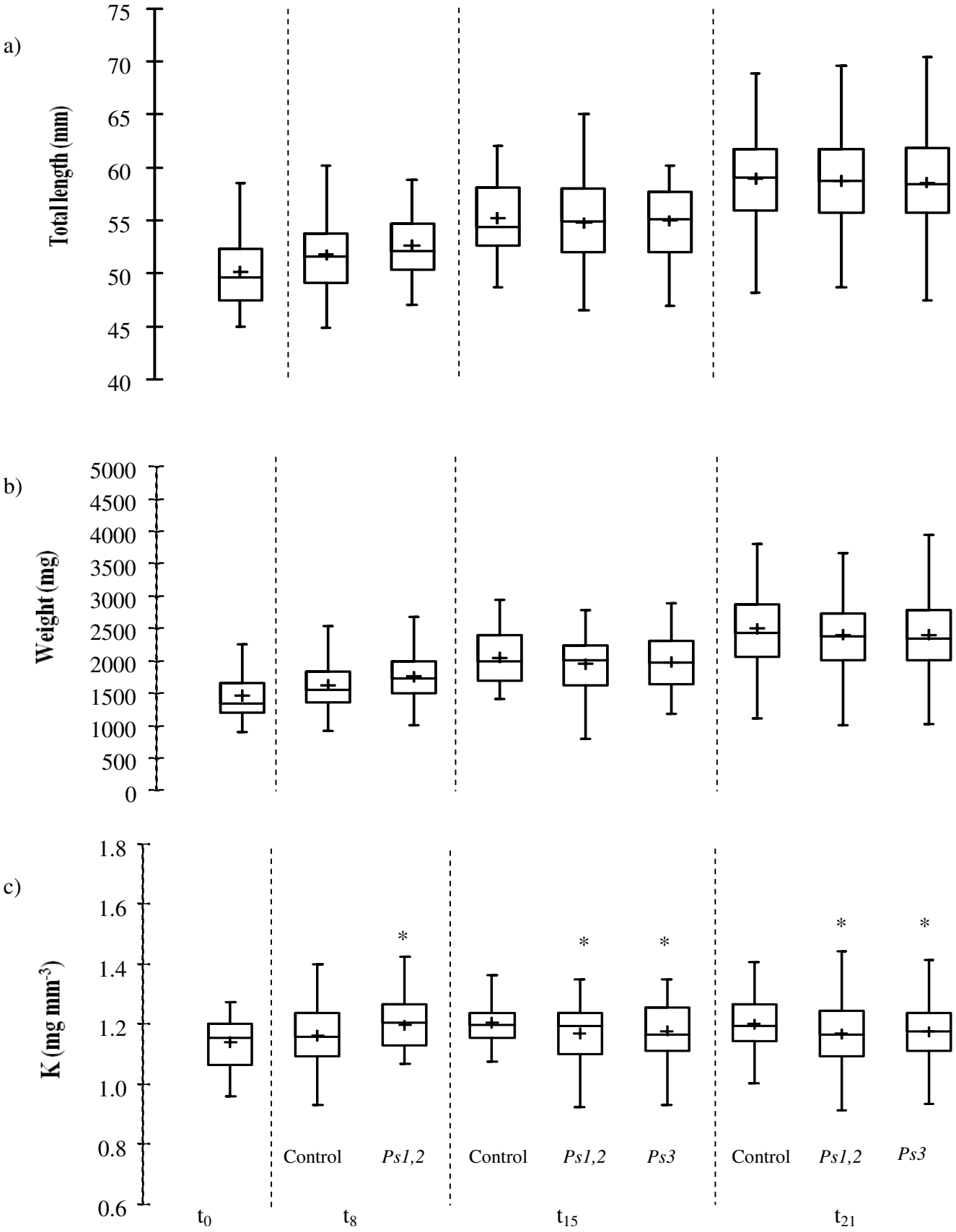


Figure 39. Box and whisker plots of sea bass juvenile size: total length (a; mm), weight (b; mg) and Fulton's condition index *K* (c; mg mm$^{-3}$) in control and two *Pseudo-nitzschia pseudodelicatissima* treatments (*Ps1,2*: exponential phase and *Ps3*: senescent phase) for each sampled week ($t_0$, $t_8$, $t_{15}$ and $t_{21}$). Whiskers extend to the highest and lowest values. Median (-) and arithmetic mean (+) are also indicated. * denotes a significant effect of treatment compared to control (KW, $p < 0.05$)

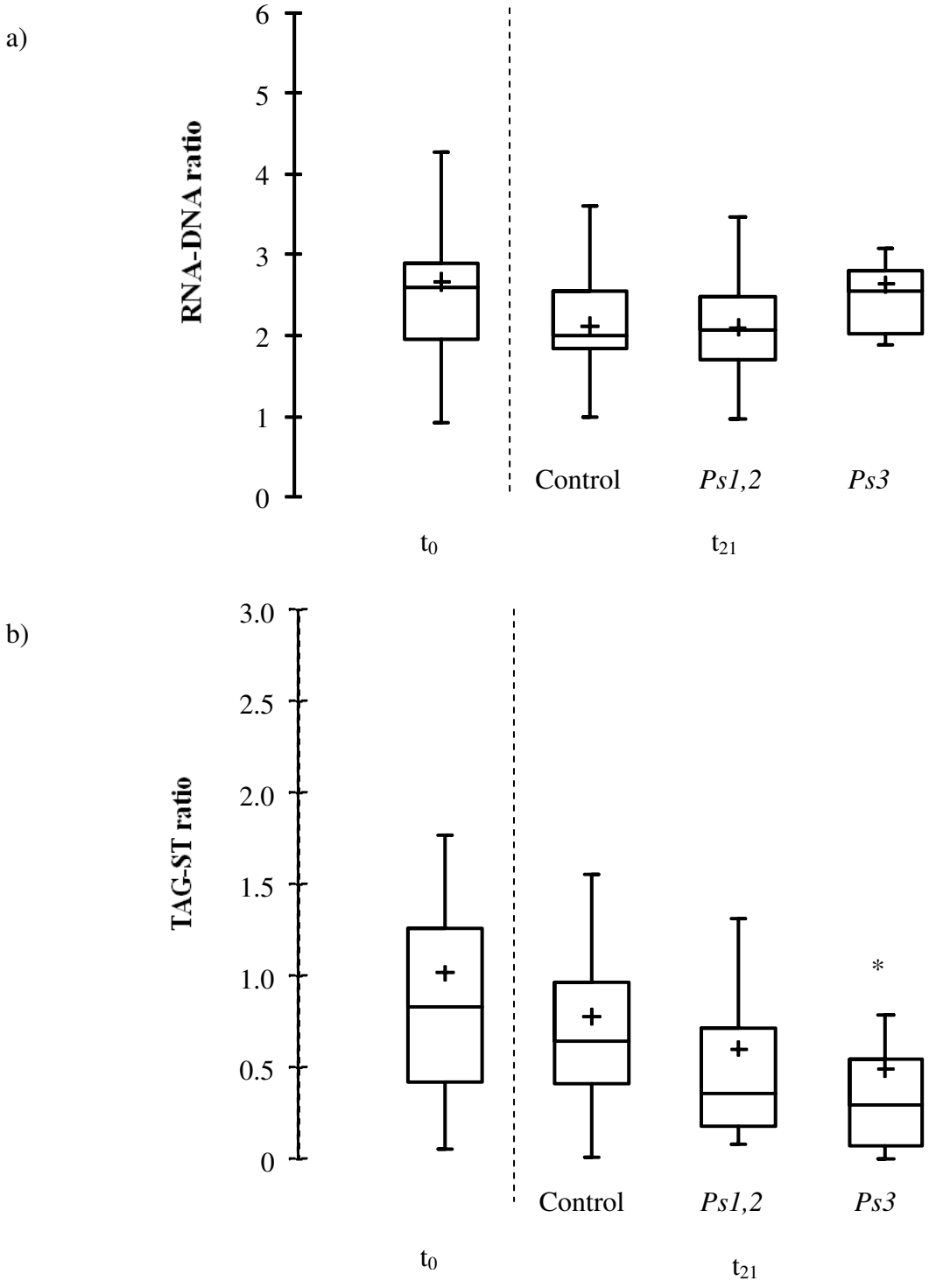


Figure 40. Box and whisker plots of sea bass juvenile size: total length (a; mm), weight (b; mg) and Fulton's condition index *K* (c; mg $mm^{-3}$*)* in control and two *Pseudo-nitzschia pseudodelicatissima* treatments (*Ps1,2*: exponential phase and *Ps3*: senescent phase) for each sampled week ($t_0$, $t_8$, $t_{15}$ and $t_{21}$). Whiskers extend to the highest and lowest values. Median (-) and arithmetic mean (+) are also indicated. * denotes a significant effect of treatment compared to control (KW, $p < 0.05$)

Table 15. Mean (± SD) values of biological parameters measured on sea bass juveniles exposed to *Pseudo-nitzschia pseudodelicatissima* production. Depicted parameters are total length (TL, mm), growth rate in length (GL; % $d^{-1}$), body weight (mg), growth rate in weight (GW, % $d^{-1}$), Fulton's condition index (K), RNA-DNA ratio, TAG-ST ratio and mortality rate (% $d^{-1}$) for each experimental treatment. Superscript ([1]), ([2]) and ([3]) stands for significant difference ($p<0.05$) when compared to "$t_0$, "control 1" and "control 2", respectively

| Dates (days) | N | Conditions | Total length (TL, mm) | GL (% $d^{-1}$) | Body weight (W, mg) | GW (% $d^{-1}$) | K (mg $mm^{-3}$) | RNA-DNA ratio | TAG-ST ratio | Mortality rate (% $d^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| **$t_0$** | 50 | - | 50.17 ± 3.54 | - | 1466.96 ± 368.77 | - | 1.14 ± 0.09 | 2.67 ± 1.26 | 1.02 ± 0.87 | - |
| | 30 | C1 | 51.95 ± 3.38 | 0.44 | 1628.54 ± 382.65 | 0.43 | 1.14 ± 0.12 | - | - | 0 |
| | 30 | C2 | 51.53 ± 3.23 | 0.34 | 1632.29 ± 347.96 | 0.52 | 1.18 ± 0.11 | - | - | 0 |
| **$t_8$** | 30 | *Ps1* | 52.42 ± 2.80 | 0.56 | 1701.95 ± 320.29 | 1.09 | 1.17 ± 0.10 | - | - | 0 |
| | 30 | *Ps2* | 52.80 ± 3.56 | 0.64 | 1817.53 ± 396.75 | 1.86 | 1.22 ± 0.14[1,2] | - | - | 0.134 |
| | - | *Ps3* | - | - | - | - | - | - | - | 0 |
| | 30 | C1 | 54.84 ± 3.10 | 0.60 | 2007.55 ± 366.22 | 1.70 | 1.21 ± 0.08[1] | - | - | 0 |
| | 30 | C2 | 55.56 ± 3.78 | 0.68 | 2102.41 ± 488.80 | 1.93 | 1.20 ± 0.08[1] | - | - | 0 |
| **$t_{15}$** | 30 | *Ps1* | 54.91 ± 4.26 | 0.60 | 1941.56 ± 511.60 | 1.33 | 1.15 ± 0.12[2,3] | - | - | 0 |
| | 30 | *Ps2* | 54.62 ± 3.48 | 0.57 | 1966.84 ± 437.57 | 1.49 | 1.19 ± 0.15[1] | - | - | 0 |
| | 30 | *Ps3* | 54.98 ± 3.55 | 0.61 | 1985.84 ± 446.84 | 1.56 | 1.18 ± 0.12 | - | - | 0 |
| | 165 | C1 | 58.94 ± 4.45 | 0.77 | 2498.61 ± 604.83 | 2.19 | 1.20 ± 0.11[1] | 1.82 ± 0.69[1] | 0.76 ± 0.56 | 0 |
| | 165 | C2 | 58.81 ± 4.24 | 0.76 | 2278.40 ± 511.51 | 1.77 | 1.10 ± 0.08[1] | 2.42 ± 0.50 | 0.80 ± 0.70 | 0.134 |
| **$t_{21}$** | 165 | *Ps1* | 58.66 ± 4.01 | 0.75 | 2527.93 ± 567.16 | 2.26 | 1.23 ± 0.10[1,2,3] | 2.46 ± 0.76[2] | 0.74 ± 0.72 | 0 |
| | 165 | *Ps2* | 57.30 ± 4.27 | 0.63 | 2202.84 ± 514.21 | 1.59 | 1.16 ± 0.13[2,3] | 1.74 ± 0.54[1,3] | 0.47 ± 0.48[1] | 0 |
| | 165 | *Ps3* | 59.83 ± 4.49 | 0.84 | 2608.42 ± 653.16 | 2.38 | 1.19 ± 0.10[1,3] | 2.64 ± 1.46[2] | 0.50 ± 0.63[1,2] | 0 |

## IV.2.4. Discussion

Aquatic mesocosms, or experimental water enclosures, are designed to provide a limited body of water with close to natural conditions, in which environmental factors can be realistically manipulated. Mesocosm systems can be defined also as culture systems for fish larvae with a water volume ranging from 1 to 10.000 m³. Such mesocosms provide a powerful tool to link between *in situ* but often only correlative field studies on the one side, and small-scale far from natural laboratory experiments including a single or a few species only, on the other side. Thus mesocosm studies have the advantage compared to laboratory approaches that it maintains a natural community under close to natural conditions, taking into account relevant aspects from 'the real world' such as indirect effects, biological compensation and recovery, and ecosystem resilience. The mesocosm approach is therefore often considered to be the experimental ecosystem closest to the real world, without losing the advantage of reliable reference conditions and replication. Many studies used these controlled environments to examine ecosystem responses to factors such as nutrient addition and light limitation e.g. essentially factors driving phytoplankton (Li et al., 1992; Egge and Hemidal, 1994; Stephen et al., 2004) and the planktonic food web dynamics (Nowlin and Drenner, 2000; Romo et al., 2004). Mesocosm systems using macro-organisms as target species have mainly focused on exposure experiments to pollutants on either macrobenthos (Gee et al., 1985; Warwick et al., 1988) or fish (Tana et al., 1994; Bony et al., 2008). The mesocosms have been used with success for rearing a variety of species, among which sea bream *Sparus aurata* (Giannakourou, 1995 and Ben Khemis, 1997), sea bass *Dicentrarchus labrax* (Nehr et al., 1996), red porgy *Pagrus pagrus* (Ben Khemis, 1997), halibut *Hippoglossus hippoglossus* (Berg, 1997), sharpsnout sea bream *Diplodus puntazzo*, white sea bream *Diplodus sargus* and greater amberjack *Seriola dumerili* (Papandroulakis et al., 2004 and Papandroulakis et al., 2005). Few studies have considered mesocosm experiments under rearing conditions (Gyllenhamar et al., 2008; Deutsch et al., 2009) although such studies can provide new insights into fish farming management.

Our mesocosm experiment allowed the observation of juvenile survival and growth at near natural densities. As a result, responses of the juveniles to controlled alteration of their environment, such as high *P. pseudodelicatissima* concentrations mediated either from exponential and senescent phases can be extrapolated to natural systems with much greater confidence than for studies done in smaller laboratory systems. Results from our experiments

clearly allowed us that two phases of *P. pseudodelicatissima* had no negative effect on the mortality, growth performance and the general welfare of juvenile sea bass analysed under fish farming conditions.

In our study, no significant differences were found between two *P. pseudodelicatissima* states treatments. In exponential phase, in both tanks (*Ps1* and *Ps2*) *P. pseudodelicatissima* concentrations (1-9E + 06 cell $L^{-1}$) followed the same trend and increased throughout the experiment. By contrast, in thank *Ps3* (senescent phase) concentrations (1-6E + 06 cell $L^{-1}$) decreased the second week of the experiment. DA-producing *Pseudo-nitzschia spp.* are globally distributed in coastal waters and capable of achieving great abundance (>$10^7$ cell $L^{-1}$) (Hasle, 2002). Phytoplankton monitoring during 1996-1998 in Scottish coastal waters revealed *Pseudo-nitzschia* species was widespread around the west coast and the northern isles and showed that maximal population levels were up to 2E + 05 cell $L^{-1}$ (Smayda, 2006). Many HABs implicating high concentrations of *Pseudo-nitzschia* are reported in the literature. During the HB-HAB of the Prince Edward Island, Canada, in 1987, *Pseudo-nitzschia* abundances reached 15E + 06 cell $L^{-1}$ (Bates et al., 1998); in Monterey Bay, USA, in 1991 *Pseudo-nitzschia* abundances were higher than $10^6$ cell $L^{-1}$ (Walz et al., 1994); and in San Diego, USA, *Pseudo-nitzschia* abundances were 7E + 04 cell $L^{-1}$. Trainer et al. (2002) found *Pseudo-nitzschia spp.* total abundances (*P. pseudodelicatissima* and/or *P. delicatissima* dominant) between 600 – 900E + 03 cell $L^{-1}$ in July 1997 and 100 - 17.000E + 03 cell $L^{-1}$ in June-October 1998 at surface water in the Washington coast. The maximum abundance of *Pseudo-nitzschia* found in this study (1-9E + 06 cell $L^{-1}$) was relatively higher compared to the abundance found in the Baie des Veys in Normandy (western part of the Baie de Seine, North of France) (9 – 38E + 03 cell $L^{-1}$) (Klein et al., 2010). This may assume adequate *P. pseudodelicatissima* production in exponential and death phase to determine their effects on the physiological performances of sea bass juveniles. Nevertheless, it was observed high mortality with higher total abundance of *P. pseudodelicatissima* during the period of end April-middle May 2009 in fish farm basins of Aquanord (Graveline, France). Mortality levels varied from 0 to 0.47 (% $d^{-1}$) and the mean values was 0.06 ± 0.13 (% $d^{-1}$). *P. pseudodelicatissima* abundance indicated the highest value (4.79E + 05 cell $L^{-1}$) when the mortality reached 0.47 (% $d^{-1}$) during the end of April and the lowest value (1.20E + 05 cell $L^{-1}$) when the mortality started to decrease in the middle of May (Figure 41). For this reason, we doubted that the effect of mortality on sea bass juvneiles could be due to the effect of *P. pseudodelicatissima.* However, in our experiment, high *P.*

*pseudodelicatissima* concentrations did not seem to negatively impact on juvenile fish. In fact, mortality was low and occurred mainly at the beginning of the experiment when *P. pseudodelicatissima* concentrations were low. Thus, our hypothesis about the mortality seems to be rejected. In addition, no morphological abnormalities or lesions were observed in the tanks during the exposition to the two physiological states of *P. pseudodelicatissima*.

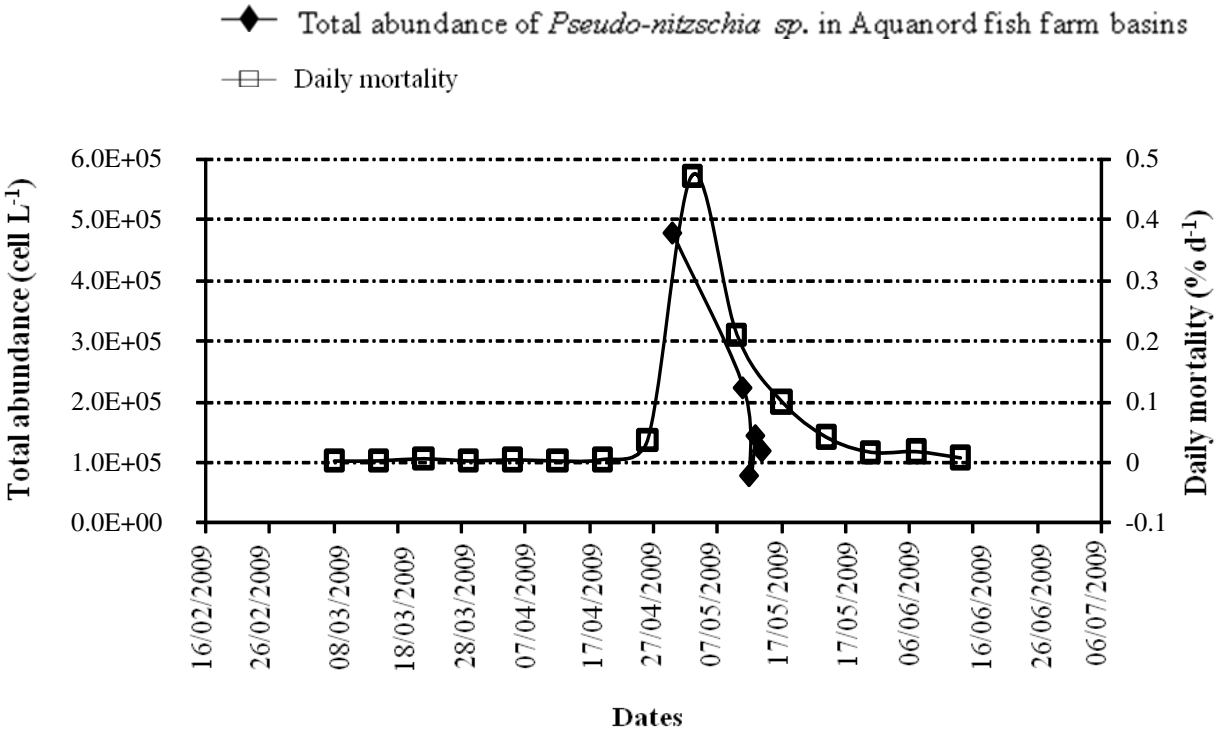


Figure 41. *Pseudo-nitzschia pseudodelicatissima* abundance (cell $L^{-1}$) in Aquanord fish farm basins and mortality rate (% $d^{-1}$) before experiment

Over the experiment, hydrological conditions (temperature, salinity, dissolved oxygen, turbidity) were similar to those observed in the English Channel when juvenile sea bass use shallow coastal nursery grounds (Selleslagh and Amara, 2008a, b). In addition, measured physico-chemical parameters appeared to be favorable for juvenile sea bass growth. In fact, specific growth rates in length (GL; 0.34 to 0.84 % $d^{-1}$) and weight (GW; 0.43 to 2.38 % $d^{-1}$) were within the range of published values for food replete fishes varying from 0.2 to 0.4 % $d^{-1}$ GL ( Millot et al., 2008) and from 2.2 to 4.55 % $d^{-1}$ GW (Hatziathanasiou et al., 2002; Valente et al., 2007). Eroldoğan et al. (2004) found higher GW (1.7 ± 0.9 % $d^{-1}$) on sea bass juveniles fed at 2 % bw $day^{-1}$ for 60 days in reared farm conditions. However, this difference could be explained by *ad libitum* feeding used in our study and the longer experiment time.

During the 21 days of experiment, juvenile sea bass size increased by 8.5 mm (0.41 mm $d^{-1}$) and their weight by 956 mg (45.54 mg $d^{-1}$). Ré et al. (1986) obtained a growth rate of 0.24 mm $d^{-1}$, using otolith daily increment analysis, in captive juvenile sea bass. Our growth rates are congruent with those recorded for wild juvenile sea bass in Portuguese estuaries (0.48 - 0.51 mm $d^{-1}$; Vinagre et al., 2009a, b; Vasconcelos et al., 2009) and near Marseille (France; 0.25 mm $d^{-1}$; Guérin-Ancey, 1973). In the Eastern English Channel the growth rate was found to be about 0.32 mm $d^{-1}$ (Selleslagh et al., 2009). This suggests that mesocosms of $1m^3$ volume are sufficient to study juvenile fish at low density since they have negligible effects on both fish growth and mortality. Data from our experiments can therefore be considered robust and relevant as rates obtained from mesocosms are close to real-life conditions.

In all treatments, the condition factor K was slightly higher in our experiment (1.10 to 1.23 mg $mm^{-3}$) compared to wild juvenile sea bass (0.88 - 1.22 mg $mm^{-3}$; Vasconcelos et al., 2009; Fonseca et al., 2011). This suggests a better feeding of fish. Since *P. pseudodelicatissima* concentrations varied between the treatments and throughout the experiment it is likely that these particles played a role in enhancing juvenile performance. In fact, higher TL, weight and/or K index were recorded when *P. pseudodelicatissima* colonies were added to mesocosms (Figure 39; Table 15).

Measures of fish growth and condition used in the present study are very sensitive to the fish environment and have thus been frequently used to assess habitat quality (*e.g*. Phelan et al., 2000; Fonseca et al., 2006; Amara et al., 2009). Nucleic acid quantification and subsequent RNA–DNA ratios have been used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Gwack & Tanaka, 2001). In all treatments, at day 21 (end of experiment), a length and weight gain was recorded, indicating that fishes globally adapted to their environment, and particularly, when *P. pseudodelicatissima* colonies were added to mesocosms (Figure 40a).

Lipides indices have been used to evaluate environmental stressor effects in fishes (Alquezar et al., 2006). In this study, the lipids storage index (TAG-ST) was used based on the ratio of the quantity of triacylglycerols (TAG; reserve lipids) to the quantity of sterols (ST; structural lipids) in the fish. The TAG content is dependent on the nutritional state of the fish as they are a principal reserve of energy in teleosts and the first components to be mobilised during periods of stress, while sterol contents remain essentially unchanged during starvation (Amara et al., 2007). The decrease of the lipid index on sea bass juveniles shows that juvenile fish appears to have depleted energy reserved in *Ps3* tank (senescent phase) at

the end of experiment (t21) compared to other treatments (Figure 40b). Lipid depletion has been identified as a general metabolic response to stress (Claireaux et al., 2004). The TAG-ST ratio of this study (0.47-1.02) were within the range reported on juvenile sole (*Solea soela*) of the Eastern English Channel (0.29-2.72) (Amara et al., 2007).

The nature of the global harmful algal blooms (HABs) problem in estuarine and coastal waters has changed considerably over the last several decades, both in extent and its public perception. Virtually every coastal country is now threatened by multiple harmful or toxic algal species, often in many locations and over broad areas (Anderson et al., 2002). Furthermore, one fo this potentiel toxic HB-HABs, *Pseudo-nitzschia spp*, which are globally distributed (Bates et al., 1998) and capable of forming extraordinarily dense blooms (Dortch et al., 1997), can be the reason of deaths of marine mammals, fish kills and outbreaks of shellfish poisonings (Masó and Garcés, 2006). In France in 2000, marine farming was closed for several weeks due to the presence of the toxin of *Pseudo-nitzschia pseudodelicatissima* and *multiseries* in shellfish (Amzil et al., 2001) and in 2004 king scallop harvesting sites in eastern English Channel were closed for several months due to contamination up to 20 mg domoic acid $g^{-1}$ tissue and to slow toxin depuration of the scallops (Nezan et al., 2006). Rhodes et al. (1998) reported also that *Pseudo-nitzschia pseudodelicatissima* has caused domoic acid contamination of shellfish in Atlantic Canada, resulting in closure of shellfish beds. Furthermore, the shallow and sheltered coastal marine areas such as bays and estuaries supported to a variaty of marine mammals and fish populations are effected by this events. Many juvenile fish and crustacean life cycles have served shallow coastal zones as nursery habitats for growth and reproduction during the warmest months of the year. Meanwhile, this periods form optimal conditions for the development of algal blooms such as *Pseudo-nitzschia pseudodelicatissima*. For this reason, this co-occurrence may affect the survival and recruitment of juvenile's fish populations (Pitmann and Pitmann, 2005). Thus, HABs can also cause mechanical damage and affect physiological processes or lead to direct pathologies in marine organisms (Landsberg et al., 2005). Their relative importance starts to be a subject of active research in the field and the laboratory. In this context, the worldwide occurrence of harmful algal blooms makes it necessary to perform environmental risk assessments to monitor the effects of this algae bloom on fish (Mazmanci and Cavaş, 2010). In recent studies, it has been focussed generally on the effects of harmful algal blooms to changes in water quality, detecting and characterising toxins, determining causative factors and developing monitoring programmes to protect human health (Pitmann and Pitmann, 2005).

In this context, Phycotoxins such as *Pseudo-nitzschia pseudodelicatissima* blooms or DA-producing diatoms can have a direct effect on fish species, causing larval and adult massive death (Trainer et al., 2007, 2008). Anyway, they can also have some important effect linked to long term accumulation of the toxins, turning them poisonous for consumers, being the humans or animals. A real accumulation of toxins hardly occurs, as the toxicity of phycotoxins to fish is quite high, so in many cases fish die before they can accumulate discrete amounts of toxins. It was also demonstrated that fish can accumulate considerable amounts of DA by dietary consumption of DA produced by toxigenic *Pseudo-nitzschia* species during or after harmful algal blooms (Landsberg, 2002). So, when accumulation occurs, liver and digestive tract is main target of accumulation. In the case of paralyzing toxins, altered swimming, equilibrium loss and complete immobility have been observed; if fish survive, recovery is complete (White, 1980; White, 1984; Carreto et al., 1993). Being toxins stored in liver and digestive tract, consumption of whole fish, as happens in Borneo and Philippines, can produce deadly episodes, as registered in past years (Maclean, 1979; Maclean, 1989). Many fish species have proved to accumulate toxins in their body: mackerels, *Sardinella* sp., *Mugil* and *Sillago*; one of the most known species which are able to accumulate toxins are puffer fish, which can stock tetrodotoxin in their viscera. Some doubt exist regarding brevetoxins capacity of accumulate; poisoning episodes in marine mammals seems to confirm the transfer of brevetoxins via plankton-eating fish (Beales, 1976; Estudillo and Gonzales, 1984; Bourdeau et al., 2001). All species able to accumulate toxins seems to have some adaptation to the poison; it the case of puffer fish and tetrodotoxin. Indeed, puffer fish have developed resistance to the toxin by a mutation of proteic sequence of sodium channel, which is the target of the toxin (Nakamura et al., 1984).

As a conclusion, the results obtained in the present study clearly showed that *Pseudo-nitzschia pseudodelicatissima* have no negative effect on juvenile sea bass. Surprisingly, *Pseudo-nitzschia pseudodelicatissima* concentrations in senescent phase had rather a positive impact on juvenile sea bass improving their physiological performance, hence, their survival and recruitment success. The work reported here need to be supplemented by a study of the measurement DA in the water and in the fish tissue and an analysis of stomach content of fish. The quantification of DA in the water will allow following the concentration during the experiment and the measurement in fish tissue will allow comparing the bioaccumulation from water into the tissue and comparing with the other treatments which has no DA. The stomach analysis may help to give information about the food selection of juvenile's fish.

Hence, more investigations are needed about the *Pseudo-nitzschia pseudodelicatissima* bloom effects on fish and their growth and survival conditions.

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# CHAPTER V

# GENERAL CONCLUSION

Marine ecosystems, and especially near-shore coastal areas such as estuaries, are one of the most important resources for aquatic organisms and are typically subjected to a variety of stressors, both natural and anthropogenic, which can impair the health and fitness of resident biota. Multiple stressors including pollutants, nutrients, harmful algal blooms (HABs) and hypoxia, turbidity, suspended sediments, and altered habitat and hydrologic regimes can impact resources through single, cumulative, or synergistic processes (Adams, 2005). These resources support also human uses, such as fishing, tourism, and shipping and threatened by growing populations in coastal regions. Numerous recent reports highlighted the concerns of excessive nutrient discharges and other contaminant inputs to coastal waters and the need to better understand such problems before these threats lead to deterioration of coastal ecosystems (Matthiessen and Law, 2002; Islam and Tanaka 2004; Di Leonardo et al., 2009; Teijon et al., 2010).

Organism responses to environmental stressors are the integrated result of both direct and indirect processes which can be ultimately manifested as changes in abundance, diversity, and fitness of individuals, populations, and communities and can occur on a variety of time scales (Schulte, 2007). Establishing causal relationships between stressors and effects on marine resources is difficult because of the physicochemical and biological complexity of these systems, the variety of biotic and abiotic factors that can modify responses of biota to stressors (McCarty and Munkittrick, 1996; Wolfe, 1996). Fish studies are important for coastal zones water and habitat quality evaluation and assessments of human impacts (e.g. Whitttield and Elliot, 2002; Le Pape et al., 2007; Elliot and Quintino, 2007) and fish have usually been used as bioindicators or biomonitors to observe the effects of natural and anthropogenic impacts (e.g. Amara et al., 2009; Costa et al., 2009b; Franco et al., 2010). Investigations into the effects of combinations of stressors are currently in their infancy, but it is clear that an understanding of the interacting effects of multiple stressors and how this affects and is affected by local adaptation of a species will be a critical component in the development of national environmental policies. Although the need is proven, the lack of information about the environmental stressors and effects on marine organisms in the Eastern English Channel was important where few studies have addressed this problem. Natural and anthropogenic disturbances are important in the ecosystem functioning and was used as a key input in this work. This work helped define the effects of environmental stressors on fish, in the Eastern English Channel, both in field and experimentally (microcosm and mesocosm) approaches on individual level and examine the effects of different types of pollution on fish

juveniles and evaluate their physiological performances in relation with other biological and/or biochemical indicators.

The purpose of this chapter is to synthesize the information discussed in the previous chapters of this thesis and to identify topics for future researches. The general conclusion will not resume point by point the issues presented in each chapter but will discuss some aspects which seems interesting and which make the originality of this work. Finally the conclusion will lead also to describe some perspectives.

## V.1. Pollution impact on fish

Fish using estuaries and coastal areas as nursery and spawning must cope with a complex set of interacting stressors, both natural and anthropogenic, which vary in both space and time (Schulte, 2007). This problem is particularly challenging because different stressors may affect related physiological and biochemical pathways, or invoke overlapping cellular responses. For this reason, a multi-biomarker approach to aquatic environmental monitoring can allow the assessment of whole animal response to a range of anthropogenic disturbances. Nevertheless, fish are interesting animals for the environmental studies. They can be used as a biological indicator in environmental research to identify the good parameters and to monitor fish health and habitat quality for the management of ecosystems. In this context, we used two approaches, *in situ* and a microcosm experiment, to determine fish responses to the environmental stressors such as anthropogenic influenced systems, and sediment chemical pollution.

We are interested in *situ* approach because estuarine and coastal systems are particularly used by juveniles of many fish species because of the potential advantages they provide for the growth and survival of young fish, namely, high prey availability, refuge from predators and good environmental conditions (Haedrich, 1983; Gibson, 1994). In contrast with their ecological importance, estuarine waters and sediments accumulate xenobiotics such as heavy metals and organic contaminants, which tends to degrade the quality of the remaining estuarine habitats for juvenile fishes. As a consequence, the essential nursery function of estuarine areas may be reduced by these anthropogenic disturbances (Gilliers et al., 2006; Le Pape et al., 2007; Courrat et al., 2009) and recruitment level and population size of the concerned marine species may then be dramatically affected (Peterson et al., 2000). The use of biological indicators, particularly those based on fish communities, has became a widespread method to assess anthropogenic impacts on estuaries (Whitfield and Elliot, 2002;

Harrison and Whitfield, 2006; Breine et al., 2007). In this context, the main objective of this *in situ* study was to evaluate the fish responses in two anthropogenically influenced systems. Seine estuary was chosen as heavily impacted system and Canche, Authie and Somme estuaries as less impacted and/or considered ''clean'' systems. As juvenile flounders concentrate in estuaries, we have chosen this species as a biological indicator to evaluate the quality of these estuarine habitats. The results of *in situ* study emphasized the negative impact of contaminants on the nursery function of estuaries. The Seine estuary exhibited the highest metals and PAHs contents in sediment compared to other estuaries and metal concentrations in juvenile flounder of this estuary were also significantly higher than ones collected in the less polluted estuaries. In the same way, fish growth and condition indices were significantly lower in individuals from this estuary in spite of the sufficient food availability. This study highlighted the use of fish as biological indicator and its responses to variable environmental factors as they are sensible organism to different condition of pollution mentioned in this study. All this results mentioned above, suggests that lower growth and condition indices of 0-group flounder in the Seine estuary may probably be due to other factors directly related to human activities such as chemical contamination. In fish, the tolerance to such stress is supported by mechanisms that involve significant energy costs (Newman & Unger, 2003), and limiting energy to other vital functions such as growth. Thus the low rates of growth and state of condition observed in juvenile flounder can strongly affect the post-winter survival of juveniles living in polluted estuaries, and consequently reduce the recruitment to the commercially exploitable stocks.

In this study, sediment analyses contribute information on the structure, quality and contaminants concentrations which can interact on the growth and condition of flounder juveniles by either accumulation into the body. Neverthless, measurement of growth and condition indices such as daily otolith increments, otolith perimeter-fish length ratio, Fulton's condition index, RNA-DNA ratio on flounder juveniles seems to give relative and interesting responses under a chronic pollution of different impacted estuarine systems. They contribute to determine the quality of estuarine habitats in field studies. Many *in situ* studies have reported use of growth rates and condition indices measured in many species such as sole, flounder, sea bass as indicator of habitat quality in estuarine nurseries (Phelan et al., 2000; Fukuda et al., 2001; Amara et al., 2007; Vasconcelos et al., 2009). The combined use of growth and condition indices may provide a useful tool to monitor and assess habitat quality for juvenile fishes, as well as the general ecological status of estuaries. This approach should be transfered and applied to other estuaries in France and Europe for future comparaisons. It

is essential to preserve and maintain the ecological functioning of these critical habitats for growth, migration, reproduction and therefore the management and sustainability of exploited marine resources. Thus, much research has been undertaken to identify, understand and evaluate the quality of these habitats and more extensive research is needed in order to evaluate how anthropogenic perturbation such as pollutants are detrimental to flounder populations and more generally to estuarine fishes.

In the *in situ* approach, there are many environmental factors such as hydrological parameters, habitat availability, eutrophication, food availability, predators, contaminants etc. that can impact on the physiological performance and hence recruitment and population abundance of fish juveniles. Hence, to control some of these environmental parameters such as hydrological parameters and food availability, a microcosm experiment was carried out on sea bass juveniles exposed to fresh sediment from five sites with different chemical concentrations using multi-biomarker approaches. Nevertheless, estuarine sediment contamination is receiving increasing attention from the scientific community, since it is recognized as a major source of ecosystem health stress (Caeiro et al., 2005). Furthermore, sediments in the aquatic environment have become an area of concern due to their potential for accumulating toxic compounds and acting as a secondary pollutant source to marine organisms (Claus et al., 2002). Therefore, the assessment of sediment contamination in estuaries and it biological and ecological significance is crucial for the management of estuarine ecosystems (Moreira et al., 2006) and the transfer of contaminants from sediments to biota is obviously a necessary requisite for the occurrence of toxicity. For example, many pelagic and epibenthic organisms depend on sediments as food source and breeding substrate can be influenced by sediment contamination which can cause the effects on reproductive, growth and recruitment of population (Solé et al., 2010). Beside this, controlled laboratory conditions are greatly simplistic compared to the natural environment and substantial extrapolation is required to predict effects for field populations (Chapman et al., 2002; Chapman, 2007). In this context, this chapter highlighted the importance of microcosm studies that can be very useful to study the effects of environmental disturbance such as sediment contamination on juvenile fish and allow extrapolating these results with *in situ* assays. Moreover, because of the lack of ecologically important assays for sediment toxicity evaluations in estuaries, this research have been directed to a multi-biomarker approach under controlled laboratory conditions for the improvement of ecological relevance in both sediment and sediment-water-organisms toxicity assessments in microcosm.

Fish are particularly vulnerable to sediment-associated pollutants. Fish behaviour and physiological responses to specific and multiple stressors have been extensively used to determine individual health and population status, and to assess habitat quality (e.g. Lloret and Planes, 2003; Marchand et al., 2003; Fonseca et al., 2006). Few studies have integrated indicators of exposure to contamination and effects on fish's health and condition, and most have reported unclear or limited responses of individual growth and condition to contaminant exposure (De Boeck et al., 1997; Wu et al., 2003; Humphrey et al., 2007). This study provides essential information to evaluate how estuarine sediment contamination influence juveniles fish using estuarine habitats as nursery grounds.

The results of this experiment emphasized no metal accumulation in fish gills and any significant differences on the physiological performances and immune system responses of fish juveniles could be observed after 21 days exposure. On the other hand, responses of molecular biomarkers, particularly, EROD, GST and CAT activities increase with the chemical contamination gradient after 7 days of exposure in sediment. This microcosm study confirmed the sensibility and relativity of short term molecular biomarkers responses to the chemical contamination. Although, all fish responses seem to be not clear, we observed only correlation between chemical pollution gradient and responses of molecular biomarkers in this experimental condition. The other indicators are not correlated. This may be due to the physiological biomarkers are tools in long term responses , however molecular biomarkers can give early warning signals of contaminant exposure so as our exposure duration seems to be not enough to observe any impact on the physiological performance of sea bass juveniles, this correlation can be less than the other indicators. In the literature, many researchers have reported evident fish responses under aïgue pollution on their physiological performance using growth and condition indices, molecular biomarkers in different fish species (Marr et al., 1996; Roméo et al., 1997; Riba et al., 2004; Humphrey et al., 2007). Recent work have suggested the link between biomarker responses and condition indices in sea bass and turbot caged polluted harbor (Kerambrun et al., 2011) or in controlled laboratory experiment under exposure of different sediment gradient pollution (Kerambrun et al., *in press*). Nevertheless, previous studies used individually some fishes (*Dicentrarchus labrax*, *Solea senegelansis* and *Pomatoschistus microps*) considering biomarker responses to contamination, in both laboratory (e.g. Gravato and Santos, 2003a, b; Fonseca et al., 2009; Vieira et al., 2009) and field conditions (e.g. Fernandes et al., 2007; Monteiro et al., 2007; Costa et al., 2009a, b). This variety of fish responses to biological or biochemical indicators in different pollution

conditions can be related due to the abiotic and biotic factors, feeding behavior, fish species, gender and reproductive stage.

These two studies either *in situ* and microcosm contribute the necessity and efficacy of the use of fish as biological indicator to monitor the environmental quality. The impact of pollution on fish seems to be contrast in both studies following the responses of different indicators. These two studies highlighted also the complexity of the fish responses to environmental stressor due to the many variable environmental factors *in situ* and due to the selection of fish species (pelagic or benthic) and the exposure duration in controlled laboratory assays.

Consequently, from all results and methodologies mentioned above in this chapter, it is possible to conclude *in situ* and microcosm studies allow us to make comparisons between physiological, biochemical biomarkers and immune parameters and to determine the sensibility and relativity of these biomarkers as an indicator of fish health and the quality of environment under exposure to anthropogenic influenced systems and sediment contamination containing complex mixture of pollutants.

## V.2. Effects of algal bloom

Beside the impact of pollution on fish, Harmful algal blooms (HABs) are widespread along the Eastern English Channel and may alter ecological functions of coastal zones and thus affecting nursery grounds and fish populations. Nevertheless, the effects of two recurrent harmful algal blooms: a) *Phaeocystis globosa* and its degraded form transparent exopolymeric particles (TEP) with foam accumulation and b) *Pseudo-nitzschia pseudodelicatissima* (exponential versus senescent phase) was investigated on the growth and condition of sea bass juveniles. Both mesocosm experiments exhibited any negative impact on juvenile sea bass physiological performance, hence, their survival and recruitment success. To our knowledge, there are few studies on the effects of algal bloom on fish performance. Thus, these mesocosms experiments can be useful to give information on this subject.

The results of *Phaeocystis globosa* experiment are congruent with the study carried out *in situ* by Selleslagh and Amara (2008b). These authors found that despite considerable interannual variability in the *P. globosa* spring bloom magnitude (Factor 40), no effect was observed on both fish and macrocrustacean species densities and diversity. This study may also conclude that sea bass juveniles seem to benefit directly and/or indirectly from the high TEP concentrations during our experiments due to increasing values in growth and condition

indices. This experience may allow us to extrapolate our mesocosm results into field studies for sea bass juveniles resident in their early life stages in the shallow coastal zones (e.g. nursery areas). Consequently, although direct or indirect benefits from TEP were suggested, further experiments (e.g. targeted feeding experiments) are needed to assess whether they contribute to the diet of sea bass juveniles. More considerable attention should be focused on the effects of this HB-HABs on the populations of fish. Moreover, the approach of this study may represent a substantial contribution, with regard to fish, to national and international policy instruments designed for sustainable management and conservation of the coastal zones and estuaries, generally, aquatic environments in the Eastern English Channel dude to the HB-HABs.

On the other hand, to our knowledge, *P. pseudodelicatissima* experiment is the first study devoted to evaluation of the condition indices in early life stage of fish exposed to this algae species. Our mesocosm experiment allowed us to observe juvenile fish survival and growth at near natural densities and to extrapolate their physiological performance responses under high *P. pseudodelicatissima* concentrations with much greater confidence *in situ*. Results from our experiments clearly allowed us that two phases of *P. pseudodelicatissima* had no negative effect on the mortality, growth performance and the general welfare of juvenile sea bass analysed under fish farming conditions. The work reported here need to be supplemented by DA measurement in the water and in the fish tissue and by fish stomach content analysis for future researches. The quantification of DA in the water may allow us to follow the concentration during the experiment and the measurement in fish tissue may allow us to compare the bioaccumulation from water into the tissue to determine the potential or negative effects of DA in fish juveniles. The stomach analysis may be helpful also to give information about the food selection of juvenile's. Consequently, the relative importance of this alga starts to be a subject of active research in the field and the laboratory. In this context, as HAB events can have direct negative effects on aquaculture production, fisheries, and public health, the worldwide occurrence of harmful algal blooms makes it necessary to perform environmental risk assessments to monitor the effects of this algae bloom on fish (Mazmanci and Cavaş, 2010). Therefore these short-term events reveal that it is essential to trace *Pseudo-nitzschia* species in our region with high-frequency sampling, especially during the warmer period in order to understand and anticipate massive contamination on marine organisms. Hence, more investigations are needed about the *Pseudo-nitzchia pseudodelicatissima* bloom effects on fish and on their growth and survival conditions for the sustainable fish populations stocks.

## V.3. Perspectives

This thesis has been addressed to answer many questions related to different environmental stressors on fish, either *in situ* and experimentally approaches, however certain points need to be more clarify with future works.

Our first approach in *situ* was to evaluate the effects environmental perturbations such as anthropogenic influence on the physiological performances of juveniles flounder in the four estuaries (Canche, Authie, Somme and Seine) and to further test fish responses utility as a monitoring tool for estuarine habitat quality. It could be interesting to follow this approach with the impacts of other environmental disturbances such as oil pollution (e.g. petroleum spills, oil tanker accidents), sewage effluents water and dredging discharges in this coastal zones and estuaries for marine organisms, especially commercially important fish species. This may help to determine and compare different environmental sources that can affect nursery grounds for juvenile fish. It could be necessary and useful to apply this approach in other estuaries of France and Europe of the Eastern English Channel (EEC) for future comparisons works. It could be also very interesting to monitor these natural or anthropogenic disturbances effects on different commercial fish species such as sea bass, plaice and sole abundant in estuarine nursery zones.

Another important and interesting approach *in situ* could be to study ‘’Caging technique’’ which seems be an alternative biomonitoring method of impacts of chemical pollution in developed and/or polluted commercial harbours (e.g. Boulogne, Calais, Dunkerque, Le Havre) and in estuaries (e.g. Canche, Authie, Seine) with different wild and aquaculture species (sea bass, flet, turbot). This method could integrate true ambient conditions over the chemical contamination in field studies for juvenile fish and could test fish species (benthic or pelagic) utility as bioindicator for habitat quality. Some parameters as age of fish or time of exposure can be controlled with this method, unless environmental parameters. The applications of fish caging could give also information from short-term indication of chemical exposure in ambient waters to extensive programmes of monitoring the exposure and effects of chemical contamination in aquatic habitats. This method has already been used in many ecotoxicology studies (Beyer et al., 1997; Winter et al., 2005; Lanier and Scharf, 2007; Gallucci et al., 2008; Jung et al., 2008) for juvenile fish. Caging technique could permit to access of health and fitness of individuals which have more ecological relevance with the measurement of growth and condition indices of fish caged in such contaminated area (Costa,

2004). Caging can also be used when detecting the impacts of deliberate uses or accidental spills of pesticides or related chemicals, of industrial and municipal discharges for permit-based follow-up, or as a part of evidence to support court actions. Thus far, no techniques have been rigorously harmonized or standardized nationally or internationally. Besides ecotoxicology, caging can be a useful technique in population biology to study, for instance, the foraging pressures by excluding or including animals from the surrounding feeding grounds (Henry and Jenkins, 1995). Thus, for practical purposes, the development of optimal caging techniques for long-term exposures of fish in the field has not reached academic priority. Excluding the work made by ICES (ICES, 2004), this appears also be true for organizations harmonizing and standardizing methodologies for environmental impact assessment. This may be felt unjustified, because a well-conducted field caging experiment with fish offers numerous advantages over traditional methods used in fish population surveys. The erffects of chemical contamination on fish caged juveniles (e.g. sea bass and turbot) in potential contaminated harbour of northern France was studied in the thesis of Elodie Kerambrun.

Our microcosm approach emphasized no negative effects on sea bass juveniles fish health under exposure to estuarine sediment toxicity and highlighted the importance utility of multi-biomarker approaches (physiological, molecular biomarkers, immunological) eventually molecular biomarkers. It seems to be interesting to follow this study with the contamination of polluted harbor sediment toxicity on diverse species, such as sediment-contact species, flounder and turbot juveniles or macrobenthic organisms to extrapolate laboratory results in field studies. Beside the sediment toxicity researches, it could also interesting to test hydrocarbons effects on juvenile fish on short-term and long term using different multi-biomarkers to evaluate environmental risk assessment.

The results of the mesocosm studies of this thesis based on the exposure of two recurrent HB-HABs: *Phaeocystis globosa* and *Pseudo-nitzschia pseudodelicatissima* indicated no negative effects on physiological performances of sea bass juveniles. However, there are missing points to complete for these experiments. It could be necessary further experiments targeted on feeding selection of sea bass juveniles to evaluate the effects of TEP derived from *P. globosa* bloom senecense. It could be also relevant to study as mentioned in the chapter V.4. on the effects of *Pseudo-nitzschia pseudodelicatissima* blooms on sea bass juveniles to quantify DA concentrations in the water coloumn and the fish tissue and to determine food selection of fish.

For future perspectives, it can be interesting to research the effects of contaminated food with metals or crude oil in water column on the growth and condition of important fish juveniles of EEC supporting with many multi-biomarkers. During this thesis, it has done a preliminary experiment (but cannot be presented in this thesis) to assess and compare the relationships between biomarkers (short term) and physiological performance (long term) responses on sea bass juveniles exposed to acute metals contamination (Cd, Cu, Pb and mixed of this three compounds) in the water column during 48h. The results showed low biomarkers responses and physiological indices with insufficient effects of sublethal metals contamination on sea bass juveniles. It could be also necessary and interesting to study the effects of metals contamination increasing the time of exposure more than 48h on fish juveniles from link between biomarker responses and biological indices and to reveal the value of biomarkers used for biomonitoring studies in natural environment. Another important future work can be to observe the effects of pharmaceutical substances (carbamazepine, ketoconazole, cimetidine, diltiazem) on fish as their residual compounds in aquatic environment has been generally recognized as a source of environmental pollutants (Fent et al. 2006; Li and Randak 2009). For example, it could be interesting to assess contamination of various pharmaceutical substances on fish juveniles with other biotransformation biomarkers (e.g. EROD, GST) used in ecotoxicology. A preliminary experiment was done during this thesis with the cooperation of INERIS (Unité d'écotoxicologie in vitro et in vivo, Institut National de l'Environnement Industriel et des Risques). The master student, Virgine Maes, studied on the possible link between the CYP3A catalytic activity and physiological disturbances measured on three-spined stickleback (*Gasterosteus aculeatus,* L.) exposed two pharmaceutical substances: carbamazepine (anti-epileptic) considered as CYP3A inductor and ketoconazole (fungicide) as CYP3A inhibitor during 7 and 21 days. Results indicated that CYP3A activity is quickly induced and responded in a dose-dependent manner for carbamazepine and showed oppositely proportional induction after only 7 days for ketoconazole. It was determined no correlation between biomarkers and physiological performances of fish.

Following this perspectives, this thesis permit us to understand causal relationships and the mechanistic processes between environmental stressors and effects either with two approaches, *in situ* and experimentally, on fish juveniles and to evaluate the importance of multi bioindicators approaches in the effective management of marine ecosystems. At the international level, between French and English partners, the DIESE project through the INTERREG IVA programme (2009-2013) is carrying out to determine of pertinent indicators

for environmental monitoring at the European scale along the eastern English Channel. Hence, this project will provide a huge contribution to knowledge for marine resource and environmental management in the areas under strong anthropogenic pressure. This project underlined also the necessity of a holistic or multidisciplinary approach for the durable management of global marine resources and environment. Among the DIESE project activities; main points to investigate are the effects of endocrine disruption, immunotoxicity and carcinogenicity interacting with chemical, biological, ecological approach in freshwater and marine ecosystems of the Channel. With this added focus on the scientific or wider social dimension, all parties who presently utilize the Channel will gain an enhanced appreciation of the need to work in harmony.

Consequently, the results of this thesis contributed to improve the fish responses with multi-biomarker approaches to monitor and assess the health of fish communities and fish habitat quality, as well as the general ecological status of coastal zones and estuaries against the various environmental stressors. More generally, it seems to be able to provide a solid scientific support on the impacts of environmental stressors on fish in the coastal areas of the Eastern English Channel.

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# ANNEX

# *List of Figures*

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